(72) Inventors: EVANS, Ronald, Mark ; 3702 Clark Street, San Diego, CA 92110 (US). WEINBERGER, Cary, A. ; 12620 Dalewood Drive, Silver Spring, MD 20906 (US). HOLLENBERG, Stanley, Mark ; 7894 Camino Jonate, San Diego, CA 92122 (US). GIGUERE, Vincent ; 3425 Lebon Drive, #731,

San Diego, CA 92122 (US). ARRIZA, Jeffrey, Louis ; 131 Redwood, Carlsbad, CA 92008 (US). THOMPSON, Catherine, Caroline ; 3903 Miramar Street, La-Jolla, CA 92037 (US). ONG, Estelita, Sebastian ; 6307 Hannon Court, San Diego, CA 92117 (US).
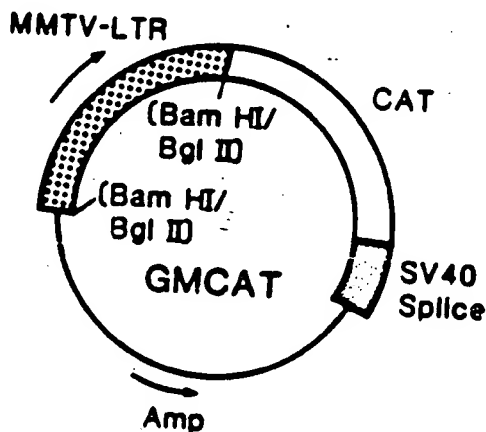
(54) Title: HORMONE RECEPTOR COMPOSITIONS AND METHODS



(57) Abstract

Substantially pure DNA and plasmids containing the DNA which is comprised of sequences which encode proteins having hormone-binding and/or transcription-activating characteristics of a glucocorticoid receptor, a mineralocorticoid receptor, or a thyroid hormone receptor. The invention further provides receptor proteins and modified functional forms thereof. The invention also provides a bioassay for determining the functionality of a receptor protein and new methods for producing desired proteins in genetically engineered cells. One method involves inducing transcription of a gene whose transcription is activated by hormones complexed with receptors: the second is a method for engineering a cell and increasing and controlling production of a protein encoded by a gene whose transcription is activated by hormones complexed with receptor proteins.

# HORMONE RECEPTOR COMPOSITIONS AND METHODS

## RELATED APPLICATION

This is a continuation-in-part of U.S.S.N. 922,585, filed October 24, 1986.

## ACKNOWLEDGMENT

## FIELD OF THE INVENTION

The present invention relates to hormone receptor proteins and genes encoding them, modification of such receptors and genes by recombinant DNA and other genetic engineering techniques, plus uses of such receptors and genes, both unmodified and modified. More particularly, the invention concerns steroid and thyroid hormone receptors and associated genes. Most particularly, it concerns human glucocorticoid, mineralocorticoid and thyroid hormone receptors and genes for them. In addition the invention relates to a novel bioassay system for determining the functionality of hormone receptor proteins coded for by receptor DNA clones, plus novel methods for inducing and controlling expression of genes whose transcription is activated by hormones complexed with receptor proteins.

## BACKGROUND OF THE INVENTION

Transcriptional regulation of development and homeostasis in complex eukaryotes, including humans and other mammals, birds, and fish, is controlled by a wide variety of regulatory substances, including steroid and thyroid hormones. These hormones exert potent effects on development and differentiation in phylogenetically diverse organisms and their actions are mediated as a consequence of their interactions with specific, high affinity binding proteins referred to as receptors. See generally, Jensen, et al., (1972); Gorski, et al., (1976); Yamamoto, et al., (1976); O'Malley, et al.,

(1969); Hayward, et al., (1982); and Ashburner, et al., (1978).

Receptor proteins, each especially specific for one of the several classes of cognate steroid hormones (i.e., estrogens (estrogen receptor), progestogens (progesterone receptor), glucocorticoids (glucocorticoid receptor), androgens (androgen receptor), aldosterones (mineralocorticoid receptor) or for cognate thyroid hormones (thyroid hormone receptor), are known and distributed in a tissue specific fashion. See Horwitz, et al., (1978) and Palmiter, et al., (1976).

Turning now to the interaction of hormones and receptors, it is known that a steroid or thyroid hormone enters cells by facilitated diffusion and binds to its specific receptor protein, initiating an alosteric alteration of the protein. As a result of this alteration, the hormone/receptor complex is capable of binding to certain specific sites on chromatin with high affinity. See Yamamoto, et al., (1972) and Jensen, et al., (1968).

It is also known that many of the primary effects of steroid and thyroid hormones involve increased transcription of a subset of genes in specific cell types. See Peterkofsky, et al., (1968) and McKnight, et al., (1968). Moreover, there is evidence that activation of transcription (and, consequently, increased expression) of genes which are responsive to steroid and thyroid hormones (through interaction of chromatin with hormone receptor/hormone complex) is effected through binding of the complex to enhancers associated with the genes. (See Khoury, et al., 1983.)

In any case, a number of steroid hormone and thyroid hormone responsive transcriptional control units, some of which have been shown to include

enhancers, have been identified.  These include the
mouse mammary tumor virus 5'-long terminal repeat (MTV
LTR), responsive to glucocorticoid, aldosterone and
androgen hormones; the transcriptional control units

5   for mammalian growth hormone genes, responsive to
glucocorticoids, estrogens, and thyroid hormones; the
transcriptional control units for mammalian prolactin
genes and progesterone receptor genes, responsive to
estrogens; the transcriptional control units for avian

10  ovalbumin genes, responsive to progesterones; mammalian
metallothionein gene transcriptional control units,
responsive to glucocorticoids; and mammalian hepatic
$alpha_{2u}$-globulin gene transcriptional control units,
responsive to androgens, estrogens, thyroid hormones

15  and glucocorticoids.  (See the Introduction portion of
Experimental Section I of this Specification for
references.)

A major obstacle to further understanding and
more practical use of the steroid and thyroid hormone

20  receptors has been the lack of availability of the
receptor proteins, in sufficient quantity and
sufficiently pure form, to allow them to be adequately
characterized.  The same is true for the DNA gene
segments which encode them.  Lack of availability of

25  these DNA segments has prevented in vitro manipulation
and in vivo expression of the receptor-coding genes,
and consequently the knowledge such manipulation and
expression will yield.

The present invention is directed to

30  overcoming these problems of short supply of adequately
pure receptor material and lack of DNA segments which
encode the receptors.

### REFERENCE LIST

The Background section of the specification

35  refers to the following publications.

## PUBLICATIONS

1.    Asburner, M., and Berenc.. , H.D. in The Genetics and Biology of Drosophila, Eds. Ashburner, M., and Wright, T.R.F., Vol. 2, pp. 315-395, Academic, London (1978).

2.    Gorski, J., and Gannon, F., A. Rev. Physiol., 38:425-450 (1976).

3.    Hayward, M.A., Brock, M.L. and Shapiro, D.J., Nucleic Acids Res., 10:8273-8284 (1982).

4.    Horwitz, K.B., and McGuire, W.L., J. Biol. Chem., 253:2223-2228 (1978).

5.    Jensen, E.V., and DeSombre, E.R., A. Rev. Biochem., 41:203-230 (1972).

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8.    McKnight, G.S., and Palmiter, R.D., J. Biol. Chem., 254:9050-9058 (1968).

9.    O'Malley, B.W., McGuire, W.L., Kohler, P.O., and Kornman, S.G., Recent Prog. Horm. Res., 25:105-160 (1969).

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11.    Peterkofsky, B., and Tomkins, G., Proc. Natl. Acad. Sci. U.S.A., 60:222-228 (1968).

12.    Yamamoto, K.R., and Alberts, B.M., A. Rev. Biochem., 45:721-746 (1976).

13.    Yamamoto, K.R., and Alberts, B.M., Proc. Natl. Acad. Sci. U.S.A., 69:2105-2109 (1972).

## OTHER PUBLICATIONS

Some of the information disclosed in this specification has been published:

The study disclosed in Experimental Section I
has been published as:  Bollenberg. S.M., Weinberger,
C., Ong, E.S., Cerelli, G., Oro, A., Lebo, R.,
Thompson, E.B., Rosenfeld, M.G., and Evans, R.M.,

5      "Primary Structure and Expression of a Functional Human
Glucocorticoid Receptor cDNA", Nature (London),
318:635-641 (December, 1985).

The study disclosed in Experimental Section II
has been published as:  Giguere, V., Bollenberg, S.M.,

10     Rosenfield, M.G., and Evans, R.M., "Functional Domains
of the Human Glucocorticoid Receptor", Cell, 46:645-652
(August, 1986)

The study disclosed in Experimental Section
III has been published as:  Weinberger, C., Thompson,

15     C.C., Ong, E.S., Lebo, R., Gruol, D.J., and Evans,
R.M., "The c-erb-A Gene Encodes a Thyroid Hormone
Receptor", Nature (London), 324:641-646 (December,
1986).

The study disclosed in Experimental Section IV

20     has been published as:  Arriza, J.L., Weinberger, C.,
Cerelli, G., Glaser, T.M., Handelin, B.L., Houseman,
D.E., and Evans, R.M., "Cloning of Human
Mineralocorticoid Receptor Complementary DNA:
Structural and Functional Kinship with the

25     Glucocorticoid Receptor", Science, 237:268-275 (July,
1987).

The study disclosed in Experimental Section V
is in press as: Giguere, V., Yang, N., Segui, P., and
Evans, R.M., "Identification of a New Class of Steroid

30     Hormone Receptors".

35

The study disclosed in Experimental Section VI is in press as: Glass, C.K., Franco, R., Weinberger, C., Albert, V.R., Evans, R.M., and Rosenfeld, M.G., "A c-erb-A Binding Site in the Rat Growth Hormone Gene Mediates Transactions by Thyroid Hormone".

The study disclosed in Experimental Section VII has been published as: Thompson, Catherine C., Weinberger, Cary, Lebo, Roger, and Evans, Ronald M., "Identification of a Novel Thyroid Hormone Receptor Expressed in the Mammalian Central Nervous System", Science, 237:1610-1614 (September, 1987).

## BRIEF DESCRIPTION OF THE DRAWINGS

The following is a brief description of the drawings. More detailed descriptions are found in the Experimental Sections of this sepcification. To avoid confusion, drawings which are referred to in Experimental Section I are labeled with the prefix "I"; those referred to in Experimental Section II are labeled with the prefix "II", and so on.

The drawings comprise 40 Figures, of which:

### EXPERIMENTAL SECTION I

FIGURE I-1 (A and B) is a drawing which shows the human glucocorticoid receptor cDNA sequencing strategy (Fig. I-1(A)), plus a schematic representation of cDNA clones (Fig. I-1(B)).

FIGURE I-2 is a drawing which shows the cDNA and predicted primary protein sequence of human glucocorticoid receptor (hGR). (Figure I-2 is divided into two parts: Fig. I-2(1) and Fig. I-2(2).)

FIGURE I-3 (A and B) is a drawing which shows the restriction map (Fig. I-3(A)) and nucleotide sequence (Fig. I-3(B)) of the 3' end of the human glucocorticoid receptor beta cDNA (beta-hGR).

FIGURE I-4 (A and B) relates to an immunoblot

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comparison of hGR translated in vitro with in vivo hGR
from cell extracts. Fig. I-4(A) is a drawing showing
the vectors constructed for in vitro transcription of
the hGR cDNA sequence. Fig. I-4(B) is a photograph
showing a Western blot analysis of in vitro translation
products and cell extracts.

FIGURE I-5 is a graph showing steroid-binding
of alpha-hGR (GR107) translated in vitro.

FIGURE I-6 (A and B) is a schematic drawing of
expression plasmids pGERR1 (Fig. I-6(A)) and pGERR2
(Fig. I-6(B)). Plasmid pGERR1 was used to express
estrogen related receptor hERR1; pGERR2 was used to
express hERR2.

EXPERIMENTAL SECTION II

FIGURE II-1 is a drawing showing a schematic
representation of the hGR functional assay.

FIGURE II-2 is a photograph showing a Western
blot analysis which illustrates expression of hGR
protein.

FIGURE II-3 is a photograph of a blot which
illustrates induction of CAT activity by hGR.

FIGURE II-4 (A and B) is a graph. Fig.
II-4(A) shows the dose-response to DEX of pRShGR alpha;
Fig. II-4(B) shows the titration of pRShGRa.

FIGURE II-5 is a schematic drawing showing the
location of functional domains in hGR.

EXPERIMENTAL SECTION III

FIGURE III-1 (A and B) is a drawing which
shows (A) the restriction map and sequencing strategy,
plus (B) the nucleotide and predicted amino acid
sequence, of human placenta c-erb-A cDNA. (Figure
III-1B is divided into two parts: Fig. III-1(B)-1 and
Fig. III-1(B)-2.)

FIGURE III-2 is a drawing showing an amino
acid sequence comparison between the carboxy-terminal
portions of the v-erb-A oncogene product, the human

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placental c-erb-A polypeptide and the human glucocorticoid and oestrogen receptors.

FIGURE III-3 (A, B and C) is a photograph of a blot showing Southern analysis and chromosome mapping of human placental DNA with c-erb-A DNA probes. Fig. III-3(A) shows human term placental DNA digested with endonucleases and separated on an agarose gel. Fig. III-3(B) shows an analysis of a placental DNA using c-erb-A as a probe. Fig. III-3(C) shows chromosome mapping of the human c-erb-A genes.

FIGURE III-4 (A and B) is a photograph showing human c-erb-A expression. Fig. III-4(A) is a blot showing a Northern analysis of RNA's from human cell lines and human placenta. Fig. III-4(B) illustrates synthesis of erb-A polypeptide in vitro.

FIGURE III-5 (A, B, C and D) shows four graphs which relate to thyroid hormone binding to erb-A polypeptides synthesized in vitro. Fig. III-5(A) is a Scatchard analysis of $^{125}I-T_3$ binding to the erb-A polypeptides made in vitro. Fig. III-5(B) shows competition of thryoid hormone analogues in vitro. Fig. III-5(C) shows competition of triiodothyronine isomers from $^{125}I-T_3$ binding to erb-A polypeptides synthesized in vitro. Fig. III-5(D) shows competition of thyroid hormone analogues for $^{125}I-T_3$ binding to 0.4 KCl HeLa cell nuclear extracts.

FIGURE III-6 is a schematic drawing which compares the steroid and thyroid hormone receptors.

FIGURE III-7 is a drawing which shows the cDNA nucleotide sequence and the predicted primary protein sequence of human thyroid receptor hERBA 8.7. (The sequence of thyroid receptor hPA 8 is related to hERBA

8.7.   See the Description of the Invention section
which follows.)

## EXPERIMENTAL SECTION IV

FIGURE IV-1 (A, B, C and D) is comprised of
three photographs and one schematic drawing, all of
which concern to isolation of a genomic sequence
related to the hGR gene.  Fig. IV=1(A) is a photograph
showing high-stringency Southern analysis of human
placenta DNA digested with the indicated nucleases.
Fig. IV-4(B) is similar except that it shows
low-stringency Southern analysis.  Fig. IV-4(C) is also
a photograph of a Southern blot; it demonstrates
isolation of the genomic sequence in a clone designated
lambda HGB.  Fig. IV-4(D) is a schematic drawing which
shows the intron-exon structure of lambda HGB genomic
fragment and its homology with hGR.

FIGURE IV-2 (A and B) is a drawing which shows
the cDNA nucleotide sequence and the predicted primary
protein sequence of human mineralocorticoid receptor.
Fig. IV-2(A) shows the composite structure of hMR
aligned with a line diagram of some restriction
endonuclease cleavage sites.  Fig. IV-2(B) shows the
complete nucleotide sequence of hMR and its primary
predicted amino acid sequence.  (Figure IV-2(B) is
divided into two parts: Fig. IV-2(B)-1 and Fig.
IV-2(B)-2.)

FIGURE IV-3 is a drawing which shows the amino
acid homology between mineralocorticoid receptor and
glucocorticoid receptor.

FIGURE IV-4 (A, B, C and D) is a drawing and
three graphs which relate to the steroid-binding
properties of expressed hMR.  Fig. IV-4(A) shows the
structure of expression plasmid pRShMr, the plasmid
used to express hMR.  Fig. IV-4(B) is a graph showing

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Scatchard analysis of tritiated aldosterone binding in
extracts prepared from pRShMR-transfected COS cells.
Figs. IV-4(C) and (D) are graphs showing competition of
unlabeled steroids for binding with [3H]aldosterone in
5    transfected COS cells.

FIGURE IV-5 (A, B and C) is a drawing and two
photographs which show transcriptional activation of
MMTV LTR by hMR and hGR expression plasmids in
transfected CV-1 cells.  Fig. IV-5(A) is a schematic
10   drawing of plasmid GMCAT.  Fig. IV-5(B) is a photograph
of a blot which shows differential CAT enzyme activity
found after hMR or hGR transfection with normal serum.
Fig. IV-5(C) is a photograph of a blot which shows
differential induction of CAT activity by aldosterone
15   or dexamethasone in cells transfected with hMR or hGR.

FIGURE IV-6 is a photograph of a blot showing
Northern analysis of mineralocorticoid receptor mRNA's
in rat tissues.

FIGURE IV-7 is a photograph showing
20   chromosomal localization of hMR gene by Southern
analysis of microcell hybrids.

FIGURE IV-8 is a schematic drawing showing
amino acid comparisons of the hGR, hMR, and hPR
structures.

25                    EXPERIMENTAL SECTION V

FIGURE V-1 (A and B) is a drawing which shows
the cDNA nucleotide sequence and the predicted primary
protein sequence of hERR1.  Fig. V-1(A) shows the
composite structure of hERR1 aligned with a line
30   diagram of some restriction endonuclease cleavage
sites.  Fig. V-1(B) shows the complete nucleotide
sequence of hERR1 and its primary predicted amino acid
sequence.  (Figure V-1(B) is divided into two parts:
Fig. V-1(B)-1 and Fig. V-1(B)-2.)

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FIGURE V-2 (A and B) is a drawing which shows the cDNA nucleotide sequence and the predicted primary protein sequence of hERR2. Fig. V-2(A) shows the composite structure of hERR2 aligned with a line diagram of some restriction endonuclease cleavage sites. Fig. V-2(B) shows the complete nucleotide sequence of hERR2 and its primary predicted amino acid sequence. (Figure V-2(B) is divided into two parts: Fig. V-2(B)-1 and Fig. V-2(B)-2.)

FIGURE V-3 is a drawing showing an amino acid sequence comparison between the carboxy-terminal regions of hERR1, hERR2, the human oestrogen and glucocorticoid receptors.

FIGURE V-4 is a photograph showing Northern blot hybridization analysis of hERR1 (Fig. V-4(A)) and mRNA's in rat and human tissues (Fig. V-4(B)).

FIGURE V-5 is a schematic drawing showing amino acid comparison between hERR1 and hERR2, hER and human thyroid hormone receptor (hT$_3$R beta).

## EXPERIMENTAL SECTION VI

FIGURE VI-1 (A, B and C) is comprised of two drawings, plus a drawing and a photograph, all of which relate to thyroid hormone responsiveness of various gene fusions containing rat GH 5'-flanking sequences. Fig. VI-1(A) is a drawing which illustrates responsiveness of 5' and 3' deletions of the rat GH gene. Fig. VI-1(B) is a drawing which shows functional analysis of the putative T$_3$ receptor binding site. Fig. VI-1(C) is a drawing/photograph combination which illustrates an mRNA transcription initiation site analysis.

FIGURE VI-2 (A and B) is a drawing which relates to binding of T$_3$ receptors to oligonucleotide probes containing biotin-11-dUTP. Fig. VI-1(A) is a

schematic representation of two oligonucleotide probes used to assay $T_3$ receptor binding to GH 5'-flanking sequences. Fig. IV-1(B) is a graph showing precipitation of $^{125}I-T_3$ labeled $T_3$ receptors from GC2 nuclear extracts by various oligonucleotide probes.

FIGURE VI-3 is a photograph showing a DNase I footprinting of the rat GH enhancer element by GC2 nuclear extracts.

FIGURE VI-4 is a graph illustrating binding to oligonucleotides containing 64 and 29 base pairs of 5'-flanking GH sequence of rat pituitary cell $T_3$ receptors and an hc-erb-A in vitro translation product.

EXPERIMENTAL SECTION VII

FIGURE VII-1 (A and B) is a schematic drawing which shows the restriction map (A), plus the nucleotide and predicted amino acid sequence (B), of thyroid hormone receptor cDNA from rat brain clone rbeAl2.

FIGURE VII-2 is a schematic drawing which compares the rat thyroid hormone receptor (rTR alpha) protein with the human thyroid hormone receptor (hTR beta) and chicken thyroid hormone receptor (cTR alpha) proteins.

FIGURE VII-3 (A, B and C) is a photograph showing Southern blot analysis and human chromosomal localization of the rTR alpha gene. Fig. VII-3(A) is a blot showing human placenta DNA hybridized to a 500-bp PvuII fragment from rbeAl2; Fig. VII-3(B) shows the placenta DNA hybridized to a 450-bp SstI fragment from hTR beta. Fig. VII-3(C) shows chromosome mapping of the rTR alpha gene.

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FIGURE VII-4 (A, B and C) shows a photograph and two graphs which relate to _in vitro_ translation and thyroid hormone binding of rTR alpha. Fig. VII-4(A) is a photograph of a SDS-polyacrylamide gel showing the _in vitro_ translation products of rTR alpha. Fig. VII-4(B) is a graph showing a Scatchard analysis of $^{125}I$-$T_3$ binding to _in vitro_ translated rTR alpha. Fig. VII-4(C) is a graph showing competition of thyroid hormone analogs for $^{125}I$-$T_3$ binding to _in vitro_ translated rTR alpha.

FIGURE VII-5 is a photograph of a gel which illustrates tissue distribution of rTR alpha mRNA.

## DEFINITIONS

In the present specification and claims, reference will be made to phrases and terms of art which are expressly defined for use herein as follows:

As used herein, GR means glucocorticoid receptor. Disclosed DNA hGR codes for glucocorticoid receptor GR.

As used herein, MR means mineralocorticoid receptor. Disclosed DNA hMR codes for mineralocorticoid receptor MR.

As used herein, TR means thyroid receptor. Disclosed human DNA's c-_erb_-A, hERBA 8.7 and hFA8, and rat rbeA12, all code for thyroid receptor.

As used herein, hERR1 and hERR2 designate DNA's which code for estrogen-related receptor proteins.

As used herein, glucocorticoid hormones include cortisol, hydrocortisone (HC), and corticosterone (CS), and analogs thereof include dexamethasone (Dex), deoxycorticosterone (Doc), and triamcinolone acetonide.

As used herein, mineralocorticoids include

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aldosterone (Aldo), as well as corticosterone (CS), and deoxycorticosterone (Doc).

As used herein, thyroid hormones include thyroxine (T4) and triiodothyronine (T3).

5   As used herein, estrogens (or oestrogens) include estradiol-17 beta, and analogs thereof include diethylstilbestrol.

As used herein, progestogens include progesterone (Prog), and analogs thereof include
10   promegestone.

As used herein, androgens include dihydroxytestosterone, and analogs thereof include methyltrienolone.

As used herein, MTV means mammary tumor virus;
15   MMTV means mouse mammary tumor virus.

As used herein, RSV means Rous sarcoma virus; SV means Simian virus.

As used herein, CAT means chloramphenicol acetyltransferase.
20   As used herein, COS means monkey kidney cells which express T antigen (Tag). See Gluzman, Cell, 23:175 (1981). COS cells are useful in the bioassay system of the present invention.

As used herein, CV-1 means mouse kidney cells
25   from the cell line referred to as "CV-1". CV-1 is the parental line of COS. Unlike COS cells, which have been transformed to express SV40 T antigen (Tag), CV-1 cells do not express T antigen. Like COS cells, CV-1 cells are useful in the bioassay system and methods of
30   the present invention.

As used herein, when it is said that a protein has "hormone-binding properties characteristic of a hormone receptor", it means that, if, in any standard assay for binding-affinity between a hormone from a
35   species, or a synthetic analog thereof, and its cognate receptor(s) from that species, the affinity of the

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protein for the hormone or synthetic analog is at least about 10% of the affinity of the hormone or analog and the cognate receptor(s) from that species.

As used herein, when it is said that the transcription-activating property of a protein (X) is "characteristic" of that of a hormone receptor (R), it means that, if, when tested in an assay such as the "cis-trans" receptor functionality bioassay system of the invention, (see Description of the Invention; also see Experimental Section II, especially Fig. II-1, plus the subsections labeled "Results" and "Experimental Procedures" which relate to use of the bioassay to show functional expression of hGR), the rate of expression from a gene (G) (whose transcription is activated by binding of: a receptor complexed with hormone or hormone analog) is, when protein (X) is employed in place of receptor (R), at least about 10% that shown when receptor (R) itself is used, as long as, in both the case of the "receptor" (R) and "protein (X)", the involved cells are bathed in the same concentration of hormone or analog thereof.

As used herein, when it is said that a protein has "hormone-binding or transcription-activating properties characteristic of a hormone receptor", it is intended that the hormone receptor itself be encompassed within this definition.

As used herein, when it is said that the transcription of a gene (G) is "substantially activated by hormone (H), or hormone analog (aH)", it means that the transcription of gene (G) is induced by binding of: a hormone/receptor [(H) or (aH)/(R) or (r)] complex to chromatin near where gene (G) is located. Under this definition (R) is meant to designate "wild-type" or unaltered hormone receptors. The lower case (r) notation is meant to designate functional "engineered" or "modified" receptor proteins, or proteins encoded by

mRNA variants of "wild-type" receptor genes.

As used herein, GRE's mean glucocorticoid
response elements and TRE's mean thyroid receptor
enhancer-like DNA sequences. GRE's are enhancer-like

5   DNA sequences that confer glucocorticoid responsiveness
via interaction with the GR. See Payvar, et al., Cell,
35:381 (1983) and Schiedereit, et al., Nature, 304:749
(1983). TRE's are similar to GRE's except that they
confer thyroid hormone responsiveness via interaction

10  with TR.

As used herein, the terms "transcriptional
control unit", "transcriptional control element",
"hormone responsive promoter/enhancer element" and "DNA
sequences which mediate transcriptional stimulation"

15  mean the same thing, and are used interchangeably.

As used herein, in the phrase "operative
hormone responsive promoter/enhancer element
functionally linked to an operative reporter gene", the
word "operative" means that the respective DNA

20  sequences (represented by the terms "hormone responsive
promoter/enhancer element" and "reporter gene") are
operational, i.e., work for their intended purposes;
the word "functionally" means that after the two
segments are linked, upon appropriate activation by a

25  hormone-receptor complex, the reporter gene will be
expressed as the result of the fact that the "hormone
responsive promoter" was "turned on" or otherwise
activated.

As used herein, the term "receptor-negative"

30  means that no receptor is detectable in the cell, or if
it is, only a de minimus amount (i.e., a barely
detectable amount) of receptor is present.

As used herein, a "mutant" of a DNA of the
invention means a DNA of the invention which has been

35  genetically engineered to be different from the
"wild-type" or unmodified sequence. Such genetic

engineering can include the insertion of new
nucleotides into the wild-type sequences, deletion of
nucleotides from the wild-type sequences, or a
substitution of nucleotides in the wild-type sequences.

5          Use of the term "substantial sequence
homology" in the present specification and claims means
it is intended that DNA or RNA sequences which have de
minimus sequence variations from the actual sequences
disclosed and claimed herein are within the scope of
10   the appended claims.

          The amino acids which comprise the various
amino acid sequences appearing herein may be identified
according to the following three-letter or one-letter
abbreviations:

15

| Amino Acid | Three-Letter Abbreviation | One-Letter Abbreviation |
|---|---|---|
| L – Alanine | Ala | A |
| L – Arginine | Arg | R |
| L – Asparagine | Asn | N |
| L – Aspartic Acid | Asp | D |
| L – Cysteine | Cys | C |
| L – Glutamine | Gln | Q |
| L – Glutamic Acid | Glu | E |
| L – Histidine | His | H |
| L – Isoleucine | Ile | I |
| L – Leucine | Leu | L |
| L – Lysine | Lys | K |
| L – Methionine | Met | M |
| L – Phenylalanine | Phe | F |
| L – Proline | Pro | P |
| L – Serine | Ser | S |
| L – Threonine | Thr | T |
| L – Tryptophan | Trp | W |
| L – Tyrosine | Tyr | Y |
| L – Valine | Val | V |

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          The nucleotides which comprise the various

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nucleotide sequences appearing herein have their usual
single-letter designations (A, G, T, C or U) used
routinely in the art.

In the textual portion of the present
specification and claims, references to Greek letters
are written as alpha, beta, etc.  In the Figures the
corresponding Greek letter symbols are sometimes used.

Expression plasmid pGEM3 is commercially
available from Promega Biotec, 2800 South Fish Hatchery
Road, Madison, WI  53711.

## DEPOSITS

Plasmids pRShGR-alpha, pRShMR, peAl01, rbeAl2
and GMCAT, all of which are in E. coli HB101, plasmids
pE4 and pHKA, both of which are in E. coli DH5, plus
plasmids phH3, phERBA 8.7 and phFA 8, have been
deposited at the American Type Culture Collection,
Rockville, Maryland, U.S.A. (ATCC) under the terms of
the Budapest Treaty on the International Recognition of
Deposits of Microorganisms for Purposes of Patent
Procedure and the Regulations promulgated under this
Treaty.  Samples of the plasmids are and will be
available to industrial property offices and other
persons legally entitled to receive them under the
terms of said Treaty and Regulations and otherwise in
compliance with the patent laws and regulations of the
United States of America and all other nations or
international organizations in which this application,
or an application claiming priority of this
application, is filed or in which any patent granted on
any such application is granted.

The ATCC Deposit Numbers for the ten deposits
are as follows:

| pRShGR-alpha | 67200 |
| pRShMR | 67201 |
| peAl01 | 67244 |
| rbeAl2 | 67281 |

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comprises a cell, preferably a mammalian cell,
transformed with a DNA of the invention. According to
this aspect of the invention, the transforming DNA is
capable of being expressed in the cell, thereby
5   increasing the amount of receptor, encoded by this DNA,
in the cell.

Further the invention comprises cells,
including yeast cells and bacterial cells such as those
of _E. coli_ and _B. subtilis_, transformed with DNA's of
10  the invention.

Still further the invention comprises novel
receptors made by expression of a DNA of the invention,
or translation of an mRNA of the invention. According
to this aspect of the invention, the receptors will be
15  protein products of "unmodified" DNA's and mRNA's of
the invention, or will be modified or genetically
engineered protein products which, as a result of
engineered mutations in the receptor DNA sequences,
will have one or more differences in amino acid
20  sequence from the corresponding naturally occurring
"wild-type" or cognate receptor (i.e., the naturally
occurring receptor of known sequence with the greatest
amino acid sequence homology to the novel receptor).
Preferably these receptors, whether "unmodified" or
25  "engineered", will have at least about 10% of the
hormone binding activity and/or at least about 10% of
the transcription-activating activity of the
corresponding naturally occurring cognate receptor.

The invention also comprises a novel method
30  for determining the functionality of hormone receptor
proteins produced from the DNA's (or mRNA's) of the
invention. The new method, which is referred to herein
as the "cis-trans" bioassay system, utilizes two
plasmids: an "expression" plasmid and a "reporter"
35  plasmid. According to the invention, the expression
plasmid can be any plasmid which contains and is

capable of expressing a receptor DNA of the invention, or an engineered mutant thereof, in a suitable receptor-negative host cell. Also according to the invention, the reporter plasmid can be any plasmid which contains an operative hormone responsive promoter/enhancer element functionally linked to an operative reporter gene.

In practicing the "cis-trans" bioassay of the invention, the expression plasmid (containing a "receptor" DNA of the invention) and the reporter plasmid are cotransfected into suitable receptor-negative host cells. The transfected host cells are then cultured in the presence and absence of a hormone, or analog thereof, which is able to activate the hormone responsive promoter/enhancer element of the reporter plasmid. Next the transfected and cultured host cells are monitored for induction (i.e., the presence) of the product of the reporter gene sequence. Finally, according to the invention, the expression and steroid binding-capacity of the receptor protein (coded for by the receptor DNA sequence on the expression plasmid and produced in the transfected and cultured host cells), is measured. (See Fig. II-2 for a schematic drawing of this "cis-trans" bioassay system.)

The "cis-trans" bioassay system is especially useful for determining whether a receptor DNA of the invention has been expressed in a transformed host cell; it is also useful in determining whether a receptor of the invention has at least about 10% of the binding activity of the corresponding naturally occurring cognate receptor, as well as whether such a receptor has at least about 10% of the transcription-activating activity of the corresponding naturally occurring cognate receptor.

Finally, it has been discovered, with the use of the DNA's of the invention, that a necessary and

sufficient condition, for activation of transcription
of a gene (G) whose transcription is activated by
hormones complexed with receptors is the presence of
the hormone and its receptor in the same cell as (G).

5  This discovery has enabled us to provide improved
compositions and methods for producing desired proteins
in genetically engineered cells.

Two of these methods are methods of the
present invention.  The first is a method for inducing

10  transcription of a gene whose transcription is
activated by hormones complexed the receptors.  The
second is a method for genetically engineering a cell
and then increasing and controlling production of a
protein coded for by a gene whose transcription is

15  activated by hormones complexed with receptor proteins.

In discussing these two methods, a gene whose
transcription is activated by hormones complexed with
receptor proteins will be referred to as gene (G).  The
hormone which activates gene (G) will be referred to as

20  (H), and any of its analogs as (aH).  Receptor protein
will be referred to as (R), and functional
modifications thereof as (r).  Finally, the cell where
gene (G) is located will be referred to as (C), and the
protein coded for by gene (G) will be referred to as

25  (P).

According to the gene induction method of the
invention, cell (C), which contains gene (G), is
transformed by a DNA of the invention, which is capable
of being expressed in cell (C) and which codes for

30  receptor (R) or a modified functional form (r) thereof;
and the concentration of hormone (H), or analog (aH),
in cell (C) is increased to a level at least sufficient
to assure induction of expression of gene (G).

According to the method for engineering a cell

35  and then producing protein (P): gene (G), which codes
for protein (P), is placed in cell (C) so that it is

under the control of a transcriptional control element
to which hormone (H), when complexed with receptor (R),
can bind, thereby inducing transcription of gene (G).
Also according to this protein production method, both

5    hormone (H) and receptor (R) are present in cell (C).
The presence of receptor (R) is assured by transforming
cell (C) with a DNA of the invention which codes for
receptor (R), or a functional modified form (r)
thereof. The presence of hormone (H), or its synthetic

10   analog (aH) is assured by bathing transformed cell (C)
in a bathing solution which contains hormone (H) or
analog (aH). Then, according to the method, the
transcription of gene (G) is controlled by controlling
the concentration of (H) or (aH) in the bathing

15   solution used to bath transformed cell (C). By so
controlling the transcription of gene (G), it is
possible to control the production of protein (P) in
cell (C).

As those skilled in the art will appreciate,
20   based on this teaching, it will now be possible to
engineer cells so that production of a protein (P),
encoded by a gene (G) whose transcription is activated
by a hormone/receptor complex, is controlled by simply
assuring the presence of hormone (H) and its receptor

25   in cell (C) where gene (G) is located, and then
controlling the concentration of hormone (H) or its
analog that is present in cell (C).

### DESCRIPTION OF THE INVENTION

The present invention relates, in part, to DNA
30   segments which code for proteins having the
hormone-binding and/or transcription-activating
properties characteristic of glucocorticoid,
mineralocorticoid and thyroid hormone receptors.
According to this aspect of the invention, these DNA

35   segments are ones capable of being expressed, in
suitable host cells, thereby producing glucocorticoid,

mineralocorticoid and thyroid hormone receptors or
receptor-like proteins.  The invention also relates to
mRNA's produced as the result of transcription of the
sense stands of the DNA's of the invention.

5          The DNA's of the invention are exemplified by
DNA's referred to herein as: human glucocorticoid
receptor DNA (hGR); human thyroid hormone receptor
DNA's (hTR: hTR alpha and hTR beta; hTR alpha is
exemplified by hERBA 8.7 and hFA 8; hTR beta is

10    exemplified by cellular or "c-erb-A"); rat thyroid
hormone receptor (rbeA12), which is the rat homolog of
human thyroid receptor alpha; human mineralocorticoid
receptor (hMR); and new human steroid hormone receptors
(hERR1 and hERR2).  The sense strand cDNA nucleotide

15    sequences, and the predicted primary protein sequences
coded for thereby, are shown in Figs. I-2(1) and I-2(2)
for hGR; in Figs. III-1(B)-1 and III-1(B)-2 for human
c-erb-A, and in Fig. III-7 for hERBA 8.7 and hFA 8; in
Figs. IV-2(B)-1 and IV-2(B)-2 for hMR; in Figs.

20    V-1(B)-1 and V-1(B)-2 and Figs. V-2(B)-1 and V-2(B)-2
for hERR1 and hERR2, respectively; and in Fig. VII-1(B)
for rat thyroid receptor rbeA12.

          DNA's hGR, human c-erb-A, hERBA 8.7, hFA8,
hMR, hERR1, and hERR2 are preferred DNA's of the

25    invention.  Also preferred are the plasmids which carry
these and other DNA's of the invention.  Preferred
plasmids include: pRShGR-alpha, pRShMR, peA101, rbeA12,
GMCAT, pE4, pHKA, phERBA 8.7, phFA 8, and phB3.

          In addition to pRShGR-alpha, preferred DNA's

30    include modifications of pRShGR-alpha which are
designated herein as I9, I37, I102, I120, I204, I214,
I262, I289, I305, I346, I384, I403, I408, I422, I428,
I440, I448, I490, I515, I532, I550, and I684, where "I"
stands for "Insert", and the number following the "I"

35    represents the DNA modification designation.  Most
preferred of the modified pRShGR DNA's are those which

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RSV-LTR-containing segment. The AA at the 3'-end of the segment shown in Fig. IV-2(B)-2 is a few bases upstream of the 5'-end of the segment which includes the SV40 polyadenylation signal.

5        Plasmid peAl01 carries the entire coding region of human thyroid receptor c-erb-A. (The gene for c-erb-A been localized to human chromosome 3; the protein product encoded by this receptor gene is now referred to as hTR beta. See Experimental Section III;

10    compare with Experimental Section VII.)

        Plasmid peAl01 was constructed by inserting the EcoRI fragment from pheAl2 (see Fig. III-1(A)) into the EcoRI site of expression vector pGEM3 (Promega Biotec), in the correct orientation. For further

15    detail on this construction see Experimental Section III, subsection III. I., under the heading labeled: Fig. III-4 Methods.

        In addition to the hTR receptor which has been localized to human chromosome 3, we have discovered a

20    second thyroid hormone receptor that is distinct from the protein sequence predicted by plasmid peAl01. We have now isolated and characterized this new and unexpected thyroid receptor from both the rat than the human. In the rat this new thyroid hormone receptor is

25    encoded by the DNA of plasmid rbeAl2. (The DNA and predicted primary protein sequence for rbeAl2 is shown in Fig. VII-1(B)) In the human, the new thyroid hormone receptor is encoded by plasmid clone hERBA 8.7, and its related clone hFA 8. (See Fig. III-7 for the sequences

30    of hERBA 8.7) hERBA 8.7 and hFA8 are cDNA products from the same gene. The DNA sequence of clone hFA 8 is identical with the DNA sequence for hERBA 8.7 (shown in Fig. III-7) with the following exceptions. The hFA 8 sequence is shorter than the sequence shown in Fig.

35    III-7. More specifically, nucleotides 1 a (G), through 514 an (A), of the ERBA 8.7 sequence are missing in the

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hFA 8 sequence. In addition the hFA 8 sequence has a deletion which extends from the guanine (G) at base pair position 1138 through the guanine (G) at base pair

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15

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1244. This deletion eliminates amino acids 368, a (Glu), through 406, a (Gln) from the polypeptide encoded by the hFA 8 clone. As stated above, our initial thyroid receptor has been localized to chromosome 3. As we show in Experimental Section VII, the human gene for the new thyroid hormone receptor has been localized to human chromosome 17. Rat thyroid receptor rbeAl2 represents the rat homolog of the human gene product from chromosome 17.

Because they are encoded by distinct genetic loci, the chromosome 17 gene products are now classified as hTR alpha and the chromosome 3 gene product is classified as hTR beta. Although highly related, the alpha and beta gene products contain specific changes in their primary amino acid sequence. Also, alpha and beta products display characteristically distinct patterns of expression.

The actions of thyroid hormones are widespread and dependent upon these receptors. Prior to our cloning of thyroid hormone receptor, this receptor had not been purified or biochemically characterized. Also, the scientific literature was entirely devoid of any evidence suggesting the existence of multiple thyroid hormone receptor gene products. The existence of multiple receptors will be useful as the basis for developing thyroid hormone analogs that selectively activate only one class of these receptors. This could have widespread clinical impact and thus represents an exciting and important discovery.

The initial thyroid hormone receptor we characterized was peAl2, the receptor now referred to as human thyroid receptor beta. We used this beta clone to screen, by molecular hybridization, a rat brain cDNA library for related sequences. This led to the identification of plasmid rbeAl2 and its subsequent identification as a novel thyroid hormone receptor of

the alpha class. Rat rbeA12 in turn was used as a
molecular hybridization probe to clone the human
homolog to the rbeA12 gene product. The human product
is encoded by clones hERBA 8.7 and hFA 8.

Additional thyroid receptor cDNA's (rat
thyroid receptor rbeA12, and human thyroid receptors
hERBA 8.7 and hFA8 can be expressed by inserting their
cDNA's, in the correct orientation, into expression
vector pGEM3, as was done for c-erb-A.

Turning now to plasmid GMCAT, it is a reporter
plasmid that contains the MTV LTR linked to the
bacterial gene for chloramphenicol acetyltransferase
(CAT). As a result of this linkage, use of pGMCAT
provides an enzymatic assay for assessing
transcriptional activity of the MTV promoter. Since
the MTV promoter contains several glucocorticoid
response elements (GRE's), reporter plasmid pGMCAT can
be cotransfected with expression plasmids carrying
glucocorticoid or mineralocorticoid receptor DNA's, now
known or later discovered, into suitable host cells.
(Such cotransfection is part of the receptor
"cis-trans" functionality bioassay system of the
present invention. This aspect of the invention is
discussed more fully below.) Detection of CAT activity
in the co-transfected host cells shows that the
polypeptides produced by the receptor expression
plasmids are functional, i.e., have transcription
activating properties characteristic of receptor
proteins. Plasmid pGMCAT has been deposited with the
ATCC for patent purposes; it has been accorded ATCC #
67282. (See Fig. IV-5(A)) for a schematic drawing of
pGMCAT.)

Plasmid GHCAT is an example of another
reporter plasmid which is useful in the present
invention. pGHCAT contains a portion of the growth
hormone promoter functionally linked to the bacterial

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gene for chloramphenicol acetyltransferase (CAT).
Because of this linkage, use of pGHCAT provides an
enzymatic assay for assessing transcriptional activity
of the growth hormone (GH) promoter. Since the GH
5 promoter contains a thyroid hormone response element
(TRE), reporter plasmid pGHCAT can be cotransfected
with expression plasmids carrying thyroid hormone
receptor DNA's, now known or later discovered, into
suitable host cells. (Such cotransfection is also part
10 of the "cis-trans" receptor functional bioassay system
of the present invention. This aspect of the invention
is discussed more fully below.) When pGHCAT is used
with a TR expression plasmid (which for example could
carry hTR alpha or hTR beta DNA) to cotransfect
15 suitable host cells, detection of CAT activity in the
co-transfected host cells can be used to show that the
polypeptides produced by the thyroid receptor (TR)
expression plasmids are functional, i.e., have
transcription activating properties characteristic of
20 thyroid receptor proteins.

Plasmids pE4 and pHKA relate to cDNA's which
encode an estrogen related receptor referred to herein
as hERR1. (See Experimental Section V, especially Fig.
V-1, parts A and B.) Plasmid pE4 carries the cDNA
25 segment referred to in Fig. V-1(A) as lambda hKE4; pHKA
carries the segment referred to in that same figure as
lambda hKA1. Both pE4 and phKA1 have been deposited
with the ATCC for patent purposes. The two plasmids
can be joined as follows to produce a single plasmid
30 (pGMERR1) which contains the entire coding sequence for
estrogen related receptor hERR1.

The preferred procedure for joining the two
cDNA clones pE4 and pHKA makes use of a synthetic
linker which is complementary at each end for a
35 specific restriction enzyme site present in each cDNA.
More specifically, the inserts from lambda hKA1 and

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lambda hKE4 are cloned as EcoRI fragments into the
plasmid vector pGM3 (P:omega Biotec); we named them
pGMKA and pGMKE, respectively.  Next, pGMKA is cut by
NarI and HindIII and the fragment encoding hERR1 is

5      purified from agarose gel (fragment 1).  pGMKE is cut
by DraIII and HindIII and the fragment encoding the 5'
end region of HERR1 and the vector sequences is
purified from agarose gel (fragment 2).  Thirdly, two
synthetic oligonucleotides complementary to each other

10     are synthesized.  The oligonucleotides are as follows:
Oligo I:
GTGCCTGGTGCGGTGGGAGGAAAACCAGAGTGTATGCTACAAGCAGCCGGCGGG;
Oligo II:
CGCCCGCCGGCTGCTTGTAGCATACACTCTGGTTTTCCTCCCACCGCACCAGGC-

15     ACTTT.  Finally, Fragment 1 and 2 and Oligo I and II
are ligated to each others according to standard
methods known well to those skilled in the art, and
then transformed into the bacterial stain DH5.  The
resulting colonies are screened for the DNA construct

20     referred to herein as pGMERR1.  Plasmid pGMERR1 can be
used to express hERR1.  See Fig. I-6(A) for a schematic
drawing of pGMERR1.

        Plasmid phH3 relates to clone lambda hH3 which
was isolated from a human heart lambda gt11 cDNA

25     library using a nick-translated 700-bp EcoRI-SmaI
fragment representing the 5' portion of lambda hKA1.
(Clones lambda hKE4 and lambda hKA1 were isolated from
a human kidney lambda gt10 cDNA library; see
Experimental Section V., H., especially the subsections

30     labeled as Figure V-1 and Figure V-2 Methods.)  Clone
lambda hH3 carries cDNA which codes for an estrogen
related receptor referred to herein as hERR2.  The cDNA
from phH3 can be inserted into pGM3 to create pGMERR2,
a drawing of which is shown in Fig. I-6(B).  The

35     functional and structural characteristics of
receptor-like polypeptides hERR1 and hERR2 are

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disclos⬤ and discussed in Experimen⬤al Section V.

One of the added di.. ~veries we have made employing the DNA's of the invention is the remarkable sequence homology among the various hormone receptors, within one species, and, for any particular receptor, among species. (See for example, Fig. III-2 which compares the carboxy-terminal portions of the v-erb-A oncogene product, the human placental c-erb-A polypeptide, and the human glucocorticoid and estrogen receptors; Fig. III-6 which compares the steroid and thyroid hormone receptors; Fig. IV-3 which compares the amino acid homology between mineralocorticoid receptor and glucocorticoid receptor; Fig. IV-8 which shows the amino acid comparisons between hGR, hMR, and hPR structures; Fig. V-3 which compares the carboxy-terminal regions of hERR1, hERR2, the human estrogen and human glucocorticoid receptors; and Fig. V-5 which shows the amino acid comparison between hERR1, hERR2, hER and human thyroid hormone receptor, $hT_3R$ beta.) As a result of this homology the DNA's and RNA's of the invention can be used to probe for and isolate a gene from virtually any species coding for a hormone receptor which activates transcription by binding to chromatin DNA after complexing with hormone. By so using the DNA's and RNA's of the invention, especially the preferred DNA's that have been deposited with the ATCC for patent purposes, those skilled in the art, without undue experimentation, can screen genomic libraries to find other glucocorticoid, mineralocorticoid and thyroid hormone receptors which fall within the scope of the present invention. This aspect of the invention is illustrated by our discovery of estrogen-related receptors hERR1 and hERR2 (see Experimental Section V, especially subsection A., Introduction, and subsection B., cDNA Clones for Receptor hERR1), and rat thyroid receptor and human

thyroid receptors TR alpha (see Experimental Section VII, especially subsection C., Isolation of a Second Thyroid Receptor DNA, and Fig. VII-1, parts A and B.)

5      DNA's and sense strand RNA's of the invention can be employed, in conjunction with the induction and protein production methods of the invention for example, to make large quantities of substantially pure receptor proteins. In addition, the substantially pure receptor proteins thus produced can be employed, using
10    well known techniques, in diagnostic assays to determine the presence of specific hormones in various body fluids and tissue samples.

       Further, the receptor proteins of the invention can be employed in screening for
15    receptor-agonists and receptor-antagonists by using binding assays such as the one discussed in Experimental Section III for binding $T_3$ to the receptor encoded by peA101, or in the "cis-trans" receptor functionality bioassay of the invention, which will be
20    discussed below.

       Finally, because the receptor proteins of the invention can be produced in substantially pure form they can be crystallized, and their structure can be determined by x-ray diffraction techniques. As will be
25    apparent to those skilled in the art, such determinations are very useful when engineering "synthetic" or modified receptor protein analogs.

       In addition to DNA's and RNA's, and the novel receptor proteins produced thereby, the present
30    invention discloses three general methods: one relates to a bioassay for determining the functionality of receptor proteins; the other two relate methods for inducing and controlling expression of genes whose transcription is activated by a hormone-receptor
35    complex bound to chromatin DNA. Each of the three general methods will be discussed separately.

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The new bioassay system for testing receptor
functionality, which we refer to as the "cis-trans"
bioassay system, utilizes two plasmids: an "expression"
plasmid and a "reporter" plasmid. According to the
5   invention, the expression plasmid can be any plasmid
capable of expressing a receptor DNA of the invention,
or a mutant thereof, in a suitable receptor-negative
host cell. Also according to the invention, the
reporter plasmid can be any plasmid which contains an
10  operative hormone responsive promoter/enhancer element,
functionally linked to an operative reporter gene. (See
the Definitions section of this Specification for an
explanation of the terms used herein.) The plasmids
pGMCAT and pGHCAT are examples of reporter plasmids
15  which contain an operative hormone responsive
promoter/enhancer element functionally linked to an
operative reporter gene, and can therefore be used in
the receptor functionality bioassay of the invention.
In pGMCAT, the operative hormone responsive
20  promoter/enhancer element is the MTV LTR; in pGHCAT it
is functional portion of the growth hormone receptor.
In both pGMCAT and GHCAT the operative reporter gene is
the bacterial gene for chloramphenicol
acetyltransferase (CAT).
25        In practicing the "cis-trans" receptor
functionality bioassay of the invention, the expression
plasmid and the reporter plasmid are cotransfected into
suitable receptor-negative host cells. The transfected
host cells are then cultured in the presence and
30  absence of a hormone, or analog thereof, able to
activate the hormone responsive promoter/enhancer
element of the reporter plasmid. Next the transfected
and cultured host cells are monitored for induction
(i.e., the presence) of the product of the reporter
35  gene sequence. Finally, according to the invention,
the expression and/or steroid binding-capacity of the

hormone receptor protein, or mutant thereof (coded for
by the receptor DNA sequence on the expression plasmid
and produced in the transfected and cultured host
cells), is measured.  (See Fig. II-1 for a schematic
5    drawing of this "cis-trans" bioassay system.)

When using the "cis-trans" receptor
functionality bioassay system of the invention to
determine the functionality of glucocorticoid or
mineralocorticoid receptors, in preferred forms,
10   plasmids will carry a selectable marker such as the amp
gene.  In addition, in preferred forms the reporter
plasmids will have the MTV LTR or a functional portion
of the growth hormone promoter as the hormone
responsive promoter/enhancer element.  MTV LTV is
15   preferred because it is known that glucocorticoid
hormones stimulate the rate of transcription of MTV DNA
by increasing the efficiency of transcription
initiation at a unique site within the MTV LTR.
Moreover, glucocorticoid receptors bind specifically to
20   DNA sequences mapped within the MTV LTR, and thus can
confer glucocorticoid responsiveness to a heterologous
promoter.  (See Experimental Section II, especially
subsection C.  (a), Assay System and Experimental
Design.)  It is also known that mineralocorticoid
25   receptor shows functional kinship with the
glucocorticoid receptor, and that the DNA binding
domain of hMR recognizes the MTV LTR.  (See
Experimental Section IV, especially subsection E.:
Expression and Hormone Binding, and subsection F.:
30   Transcriptional Activation).  Growth hormone promoter
is preferred because its activation is responsive to
binding by thyroid hormone-receptor complex.

Preferred host cells for use with the
"cis-trans" bioassay system of the invention are COS
35   cells and CV-1 cells.  (See Experimental Section II,
subsection C. (a) Assay System and Experimental Design,

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for use of the preferred host cells in the bioassay system of the present invention.) COS-1 (referred to as COS) cells are mouse kidney cells that express SV40 T antigen (Tag); CV-1 do not express SV40 Tag. CV-1

5 cell are convenient because they lack any endogenous glucocorticoid or mineralocorticoid or other known steroid or thyroid hormone receptors. Thus, via gene transfer with appropriate expression vectors, it is possible to convert these host cells from receptor

10 negative to receptor positive. The presence of Tag in the COS-1 derivative lines allows the introduced expression plasmid to replicate and provides a relative increase in the amount of receptor produced during the assay period.

15       Expression plasmids containing the SV40 origin of replication (ori) can propagate to high copy number in any host cell which expresses SV40 Tag. Thus our expression plasmids carrying the SV40 "ori" can replicate in COS cells, but not in CV-1 cells. Although

20 the increased expression afforded by high copy number is desired, it is not critical to the disclosed bioassay system. The use of any particular cell line as a "host" is also not critical. The expression vectors are so efficient that, in our hands, the assay

25 has worked in all the hosts we have examined. CV-1 cells are preferred only because they are particularly convenient for gene transfer studies and provide a sensitive and well-described host cell system.

      The "cis-trans" bioassay system is especially

30 useful for determining whether a receptor DNA of the invention has been expressed in a transformed host cell; it is also useful in determining whether a receptor of the invention has at least about 10% of the binding activity of the corresponding naturally

35 occurring cognate receptor, plus whether such a receptor has at least about 10% of the

transcription-activating activity of the corresponding
naturally occurring cognate receptor.

Fig. II-1 schematically illustrates use of the
"cis-trans" receptor functionality bioassay system of
5   the invention when used to determine the functionality
of receptor polypeptides coded for by hGR cDNA. Details
of the bioassay, and its effectiveness as a
quantifiable bioassay system to test receptor
functionality, are disclosed and discussed in
10  Experimental Section II.  (See especially, subsection
F., Experimental Procedures, and subsection C., (b),
Expression of Functional hGR.)  As that experimental
section shows, in addition to the CAT enzymatic assay,
which can be used to show activation of the hormone
15  responsive promoter/enhancer element, Western blot
analysis of the transfected host cells can be used to
demonstrate synthesis of receptor polypeptides which
are indistinguishable with respect to mobility from the
cognate receptors used as controls.  Moreover, by using
20  the "cis-trans" bioassay system of the invention,
activation of the receptors (produced in the
transfected and cultured host cells) by specific
hormones can also be examined, as can their
hormone-binding capabilities and characteristics.  As
25  Experimental Section II demonstrates, when this was
done for hER, it was shown that the hGR of the
invention is functional and binds with glucocorticoid
hormones with the same specificity and concentrations
as does the cognate receptor.

30      Finally, as stated in the Summary section, by
using the DNA's of the invention we have discovered
that a necessary and sufficient condition for
activation of transcription of a gene (G), whose
transcription is activated by hormones complexed with
35  receptors, is the presence of the hormone and its
receptor in the cell (C) where (G) is located.  (The

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method by which hormone (H) and receptor (R) effect
gene G's transcription is not fully understood.
However, it is believed that receptor (R), when
complexed with hormone (H), binds to specific DNA

5   sites, referred to in the art as "transcriptional
control elements" or "DNA sequences which mediate
transcriptional stimulation", which are located on the
chromatin near where gene (G) is located. This binding
by the hormone/receptor, or (H)/(R), complex seems to

10   act in a way not yet understood, as a hormone dependent
"switch" that "turns on", or in some other manner
activates, the promoter for gene (G), and thus
stimulates the transcription of the (G) gene.)

       Our discovery has enabled us to provide

15   improved compositions and methods for producing desired
proteins in genetically engineered cells. Two of these
methods are methods of the present invention. The
first is a method for inducing transcription of a gene
whose transcription is activated by hormones complexed

20   the receptors. The second is a method for engineering
a cell and then increasing and controlling production
of a protein encoded by a gene whose transcription is
activated by hormones complexed with receptor proteins.

       Again, in discussing these two methods, a gene

25   whose transcription is activated by hormones complexed
with receptor proteins will be referred to as gene (G).
The hormone which activates gene (G) will be referred
to as (H), and any of its analogs as (aH). Receptor
protein will be referred to as (R), and functional

30   modifications thereof as (r). Finally, the cell where
gene (G) is located will be referred to as (C), and the
protein coded for by gene (G) will be referred to as
(P).

       According to the gene induction method of the

35   invention, cell (C), which contains gene (G), is
transformed by a DNA of the invention, which is capable

of being expressed in cell (C) and which codes for
receptor (R) or a modified functional form (r) thereof;
and the concentration of hormone (H), or analog (aH),
in cell (C) is increased to a level at least sufficient
to assure induction of expression of gene (G).

As we show in Experimental Section II, when we
used the induction method of the invention, to our
great surprise, the presence of (H) and (R) in the cell
(C) where gene (G) was located not only induced
transcription of gene (G) but also increased production
of protein (P) 500-1000 fold. This finding showed us
that the induction method can be used to not only
induce transcription, but to increase and control it as
well. This finding also led us to develop our method
for engineering a cell and then controlling production
proteins (P) coded for by genes (G) whose transcription
is activated by hormones complexed with receptors. This
method will be discussed more fully below.

Our induction method can also be used to
increase and control production of protein (P) by
simply adjusting the concentration of hormone (H), or
analog (aH), available to cell (C). (As those skilled
in the art will understand, by transforming cell (C)
with a DNA of the invention, an adequate supply of (R)
or (r) can be assured in cell (C) so that lack of (R)
or (r) will no longer be a limiting factor in the
transcription of gene (G). This being the case, by
simply increasing the amount of (H) or (aH) in the
culture solution, it will be possible to increase
transcription of gene (G) and consequently the amount
of protein (P) that is produced in (C) cells.)

The induction method of our invention can be
used to induce expression of any gene (G) that is under
transcriptional control of a transcriptional control
element activated by binding of a steroid or thyroid
hormone receptor complexed with one of its hormones

(H), or analogs (aH) thereof, so long as: (1), a DNA is available which codes for receptor (R), or a functional modified form (r) thereof which has the transcription-activating properties of (R); (2), cell

5  (C) is a cell that can be cultured; and (3), cell (C) can be transformed to express the (R)- or (r)-coding DNA needed to complex with hormone (H) or analog (aH).

Without undue experimentation those skilled in the art can use any of the deposited DNA's of the

10  invention as probes to search genomic libraries for (R)- or (r)-coding DNA's which are not now available. Once found, these DNA's, if expressible in the cell (C) where gene (G) is located, can be used to transform (C) cells. Methods for transforming cultured cells are

15  well krown and can be used by those skilled in the are without undue experimentation. Also without undue experimentation, those skilled in the art can determine what the base level of (H) is in cell (C), if any is present, as well as what the concentration of (H) or

20  (aH) must be in order to induce and control transcription of gene (G), and thus production of protein (P). The requisite concentrations of (H) can be supplied to transformed (C) cells by adding (H) or (aH) to the culture solutions used to bath cultured (C)

25  cells.

We have taught that a necessary and sufficient condition for transcription of gene (G) is the presence of (H) or (aH) and (R) or (r) in the cell (C) where gene (G) is located, and that transcription of gene

30  (G), and therefore production of protein (P), can be induced and controlled by simply increasing the amount of (H) or (aH) in the culture solutions used to bath transformed (C) cells. As those skilled in the art will appreciate, based on these teachings, it will now

35  be possible to engineer cells so that production of a protein (P), encoded by a gene (G) whose transcription

is activated by a hormone/receptor complex, is
controlled by simply assuring the presence of hormone
(H) and its receptor in cell (C) where gene (G) is
located, and then controlling the concentration of
5    hormone (H) or its analog that is present in cell (C).
This concept is the basis for the cell engineering and
protein production method of our invention.

According to our engineered cell and protein
production method: (1), cell (C) is engineered to
10   contain gene (G) so that transcription of gene (G) is
under the control of a transcriptional control element
to which an appropriate hormone/receptor, (H)/(R),
complex can bind, thereby activating transcription of
gene (G); (2), cell (C), which now contains gene (G)
15   under the control of a transcriptional control element,
is transformed by a DNA of the invention, which is
capable of being expressed in cell (C) and which codes
for receptor (R) or a modified functional form (r)
thereof; and (3), finally the concentration of hormone
20   (H), or analog thereof, in cell (C) is adjusted so that
the transcription of gene (G) is not only induced but
effectively increased and controlled by increasing
controlling the amount of hormone (H) that is available
to cell (C) from the culture solution used to bath
25   transformed (C) cells.  By so increasing and
controlling gene (G)'s transcription, production of
protein (P) is also increased and controlled.

As with the induction method, in our
engineered cell and protein production method, both
30   hormone (H) and receptor (R) are present in cell (C).
Again, as with the induction method, the presence of
receptor (R), or a functional modified form (r) thereof
is assured by transforming cell (C) with a (R)- or
(r)-coding DNA of the present invention.  As stated
35   above, methods for transforming cultured cells are well
known and can be used by those skilled in the art

without undue experimentation. The presence of (H), or
its analog (aH), is assured, and the concentration of
(H) or (aH) is controlled, by simply bathing
transformed (C) cells in bathing solutions which

5   contain appropriate concentrations of (H) or (aH).
Appropriate concentration, i.e., concentrations of (H)
or (aH) needed to for cell (C) to produce a given
amount of protein (P) can be determined in a given
situation by those skilled in the art, without undue

10  experimentation.

Again, as those skilled in the art will
understand, by transforming cell (C) with a DNA of the
invention, an adequate supply of (R) or (r) can be
assured in cell (C) so that lack of (R) or (r) will no

15  longer be a limiting factor in the transcription of
gene (G). This being the case, by simply increasing
the amount of (H) or (aH) in the culture solution, it
will be possible to increase the amount of protein (P)
that is produced in (C) cells.

20  As was true with our induction method, the
engineered cell and protein production method of our
invention can be used to control expression of any gene
(G) that can be inserted into cell (C) so that it is
under transcriptional control of a transcriptional

25  control element activated by binding of a steroid or
thyroid hormone receptor (R) complexed with one of its
hormones (H)), or analogs thereof, as long as: (1), a
DNA is available which codes to receptor (R), or a
functional modified form (r) thereof which has the

30  transcription-activating properties of (R); (2), cell
(C) is a cell that can be cultured; and (3), the (R)-
or (r)-coding DNA is capable of being expressed in cell
(C) where gene (G) is located.

Again, without undue experimentation, those

35  skilled in the art can use any of the deposited DNA's
of the invention as probes to search genomic libraries

for (R)- or (r)-coding DNA sequences not now available. Once found, these DNA's, if expressible in cell (C) where gene (G) is located, can be used in the engineered protein production method of the present invention.

Also without undue experimentation, those skilled in the art can determine what the base level of (H) is in cell (C), if any is present, as well as what the concentration of (H) or (aH) must be in order to induce and control transcription of gene (G), and thus production of protein (P). The requisite concentration of (H) needed to assure the production of a desired amount of protein (P) can be supplied to transformed (C) cells by adding (H) or (aH) to the culture solutions used to bath cultured (C) cells.

Various aspects of the present invention are further explained and exemplified in the seven experimental sections which follow. Experimental Section I relates to human glucocorticoid receptor. More specifically, that section discloses the primary structure of hGR cDNA, as well its expression into a polypeptide which is functionally indistinguishable from previously disclosed hGR proteins. Experimental Section II relates to functional domains of hGR. As that section discloses, GR contains at least four functional domains, two of which were expected and correspond to the predicted DNA- and steroid-binding domains, and two of which were not expected, and have potent effects on transcription. Experimental Section III relates to thyroid hormone receptor c-erb-A. As that section discloses, c-erb-A encodes a thyroid hormone receptor we now refer to as hTR alpha. Taken in conjunction with our unexpected discovery of a second thyroid hormone receptor (see Experimental Section VII), the data disclosed about c-erb in Section III will be extremely useful in gaining further

knowledge about thyroid receptor  roteins. Experimental
Section IV relates to human mineralocorticoid receptor,
which we show has a structural and functional
similarity to glucocorticoid receptor.  Experimental
5    Section V discloses a new and unexpected class of
steroid hormone receptors we refer to as hERR1 and
hERR2.  These receptors provide the first evidence for
the existence of a novel steroid hormone system.  In
conjunction with our disclosure of a new "cis-trans"
10   bioassay system, the new estrogen-related hERR1 and
hERR2 receptors will provide the basis for development
of an assay system that will systematically lead to the
identification of novel hormones.  The identification
of such novel systems is likely to have widespread
15   physiologic and clinical significance.  In Experimental
Section VI we disclose some of our data relating to the
sites in a rat thyroid receptor c-erb-A oligonucleotide
which we found were necessary for $T_3$ regulation.  Such
knowledge, taken in conjunction with Experimental III
20   and our disclosure in Experimental Section VII of a new
 d unexpected thyroid hormone receptor that is linked
to human chromosome 17, will be useful in
characterizing the thyroid receptor proteins.

25           Without further elaboration, it is believed
that one of ordinary skill in the art, can, using the
preceding description, and the following Experimental
sections, utilize the present invention to its fullest
extent.  The material disclosed in the experimental
30   sections, unless otherwise indicated, is disclosed for
illustrative purposes and therefore should not be
construed as being limitive in any way of the appended
claims.


35

EXPERIMENTAL SECTION I

PRIMARY STRUCTURE AND EXPRESSION OF A

FUNCTIONAL HUMAN GLUCOCORTICOID RECEPTOR cDNA

5                           I. A.   SUMMARY

Here we report the complete amino-acid
sequence of the human glucocorticoid receptor (hGR),
deduced from human lymphoid and fibroblast cDNA clones.
The sequence reveals various structural features of the
10   receptor, including the major immunogenic domain and a
cysteine/arginine/lysine-rich region which may
constitute a portion of the DNA-binding domain.  We
describe the use of the SP6 transcription vector system
to generate analytical amounts of full-length protein,
15   and demonstrate that the cell-free translated protein
is both immunoreactive and possesses steroid-binding
properties characteristic of the native glucocorticoid
receptor.  Weinberger, et al., (1985b) describes the
homology of the hGR sequence to that of the oncogene
20   v-erb-A.

I. B.   INTRODUCTION

The glucocorticoid receptor is widely
distributed and expressed in many cultured cell lines,
and the control of gene expression by glucocorticoids,
25   therefore, has been widely studied as a model for
transcriptional regulation.  A number of
glucocorticoid-responsive transcription units,
including mouse mammary tumor virus (MMTV) (Ringold, et
al., 1975; Parks, et al., 1974), mouse and human
30   metallothionein (Hager, et al., 1981; Karin, et al.,
1980), rat alpha$_{2M}$-globulin (Kurtz, et al., 1977) and
rat and human growth hormone (Spindler, et al., 1982;
Evans, et al., 1982; Robins, et al., 1982) genes have
been identified.  DNA sequences mediating
35   transcriptional stimulation of several of these genes
have been localized.  For MMTV, these sequences are
discrete genomic regions upstream of the

transcriptional start site .. ich appear to exert their
actions independently of orientation and position
(Chandler, et al., 1983; Ostrowski, et al., 1984).  The
5   steroid/receptor complex appears to bind to these
regulatory sequences and purified receptor has been
used to define the specific binding sites(Govinda, et
al., 1982; Scheidereit, et al., 1983; Pfahl, 1982;
Payvar, et al., 1983).  Based on the footprinting
10  analyses of several responsive genes, a consensus DNA
binding sequence sharing the core sequence 5' TGT/CTCT
3' has been proposed (Karin, et al., 1984).

        The ability of the glucocorticoid-responsive
element (GRE) to alter its position and orientation yet
15  still maintain promoter inducibility suggests that it
resembles the class of cis-acting regulatory sequences
termed enhancers (Chandler, et al., 1983).  First
discovered in viruses and subsequently in cellular
genes, these sequences are necessary for efficient
20  transcription in vivo (Laimonis, et al., 1982; Benoist,
et al., 1981; Baerji, et al., 1983).  It has been
suggested that enhancers are recognized by trans-acting
factors that mediate regulatory effects by
tissue-specific transcriptional control.  Although the
25  enhancer factors have not been well characterized, the
glucocorticoid receptor may serve as a paradigm for
these putative gene activator proteins.

        The availability of radiolabeled high-affinity
glucocorticoid analogues such as dexamethasone and
30  triamcinolone acetonide has led to the development of
purification strategies resulting in the isolation of
nearly pure rat and human receptors (Simons, et al.,
1981; Gehring, et al., 1983).  Although the receptor
migrates as a dimer in sucrose gradients, analysis on
35  denaturing SDS-polyacrylamide gels detects a single
polypeptide of relative molecular mass $(M_t)$ ~94,000
(94K) (Westpahl, et al., 1982; Wrange, et al., 1979).

The native polypeptide contair- intrinsic specificity for steroid binding and DNA sequence recognition. By using as probes monoclonal and polyclonal antibodies

5    raised against the purified rat and human receptors (Okret, et al., 1981; Harmon, et al., 1984; Gametchu, et al., 1984), it has been possible to identify a major immunogenic region in the receptor residing on a portion of the molecule that is distinct from the

10   steroid- and DNA-binding regions (Carlstedt-Duke, et al., 1982; Wrange, et al., 1984; Deliweg, et al., 1982). To gain further information about the structure of this molecule and to begin an analysis of the molecular mechanisms by which it regulates gene

15   transcription, we set out to clone receptor cDNA sequences. By using receptor-specific antibodies as probes, we and others have isolated clones containing human or rat glucocorticoid receptor cDNA inserts (Weinberger, et al., 1985a; Miesfeld, et al., 1984).

20               I. C. RESULTS

(a) GLUCOCORTICOID RECEPTOR cDNA

      A library of cDNA clones was constructed in the phage expression vector lambda gt11 using poly(A)[+] RNA from the human lymphoid cell line IM-9 as template,

25   as described previously (Weinberger, et al., 1985a). This library was initially screened with a rabbit polyclonal antiserum to the purified glucocorticoid receptor, resulting in the isolation of several immunopositive candidate clones from ⁻2.5 x 10^{5}

30   plaques. The beta-galactosidase fusion proteins generated from these clones were used to affinity-purify receptor epitope-specific antibody, which was subsequently recovered and identified by binding to protein blots of cellular extracts. Three

35   clones containing inserts expressing antigenic determinants of the human glucocorticoid receptor were isolated. The inserts of these clones, although of

different sizes, cross-hybridized, indicating that they contained a common sequence which presumably delimits the major immunogenic domain of the receptor. Together, these clones spanned 1.4 kilobase pairs (kbp) but were

5    clearly not long enough to code for the entire receptor, which was estimated to require ⁻2,500 nucleotides to encode a polypeptide of $M_t$ 94K.

To isolate additional cDNA clones we again screened the original library and also examined a

10   second library (given by H. Okayama) prepared with poly(A)⁺ RNA from human fibroblasts in the vector described by Okayama and Berg (1983). Using one of the immunopositive cDNA inserts (hGR1.2) as probe, 12 clones were isolated that, together, covered more than

15   4.0 kbp. The nucleotide sequences of these clones were determined by the procedure of Maxam and Gilbert (1977) according to the strategy indicated in Fig. I-1(A). RNA blot analysis indicated that a cDNA insert of 5-7 kilobases (kb) would be necessary to obtain a

20   full-length clone and sequence analysis indicated that the overlapping clones OB7 and hGR5.16 spanned an open reading frame of 720 amino acids, not large enough to encode the complete receptor. Therefore, a second human fibroblast cDNA library of ⁻2 x 10⁶ transformants

25   was screened, yielding a clone (OB10) containing a large insert that extended 150 base pairs (bp) upstream of the putative translation initiation site (see Fig. I-1(A)). Sequence analysis predicts two protein forms, termed alpha and beta, which diverge at amino acid 727

30   and contain additional distinct open reading frames of 50 and 15 amino acids, respectively, at their carboxy termini (see Fig. I-1(B)). The alpha form, represented by clone OB7, is the predominant form of glucocorticoid receptor because eight cDNA clones isolated from

35   various libraries contain this sequence.


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## (b) cDNA AND PROTEIN SEQUENCES

Figures I-2(1) and I-2(2) show the 4,800-nucleotide sequence encoding the human alpha glucocorticoid receptor, determined using clones hGR1.2, hGR5.16, OB7 and OB10. The translation initiation site was assigned to the methionine codon corresponding to nucleotides 133-135 because this is the first ATG triplet that appears downstream from the in-frame terminator TGA (nucleotides 121-123). However, in the absence of amino-terminal peptide sequence information, unequivocal determination of the initiation site is not yet possible. The codon specifying the lysine at position 777 is followed by the translation termination codon TGA. The remainder of the coding sequence is covered by multiple overlapping clones, with OB7 containing a 4.3-kb insert that continues to the poly(A) addition site and OB10 containing the putative initiator methionine. The 3' regions of clones OB7 and OB10 diverge at nucleotide 2,314, as shown by both restriction endonuclease and DNA sequence analysis. At this junction, the alpha-receptor continues with a unique sequence encoding an additional 50 amino acids whereas the beta-receptor continues for only 15 additional amino acids (Fig. I-3(B)). The 3'-untranslated region of OB7 is 2,325 nucleotides long, while that of OB10 is 1,433 nucleotides. There is no significant homology between these two regions, as indicated by direct sequence comparison (Figs. I-2(1), I-2(2) and I-3(B)) or by hybridization analysis under stringent conditions (data not shown).

In addition, we have isolated from a human primary fibroblast library another cDNA clone, OB12 (data not shown), which contains sequences identical to OB7 but uses the polyadenylation signal at nucleotide 3,101 (Figs. I-1(B) and I-2(1) and I-2(2)), giving rise to a shorter 3'-untranslated region. Use of probes

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specific for the 3'-untranslated region of OB7 to
screen a human placenta cDNA library reveals that most
clones terminate at the first poly(A) site in OB7.
Thus, messenger RNA variation is the apparent conse-

5    quence of both alternative polyadenylation and alterna-
tive RNA splicing (see below).  The fact that the human
fibroblast library contained both cDNA's suggests that
both receptor forms may be present in the same cell.

(c) ANALYSIS OF ALPHA- AND BETA-RECEPTOR PROTEIN

10           Sequence analysis reveals that the alpha and
beta forms of the human glucocorticoid receptor are 777
and 742 residues long, respectively; the two forms are
identical up to residue 727, after which they diverge.
To examine the receptor levels in vivo, cytoplasmic

15   extracts from several human and mouse cell lines were
probed by immunoblot analysis with a polyclonal
antibody directed against the human glucocorticoid
receptor (Harmon, 1984).  Alpha- and beta-receptor
cDNA's were inserted into the SP6 transcription vector

20   to create synthetic mRNA for in vitro translation (Fig.
I-4(A)).  The RNA's were separately added to a rabbit
reticulocyte lysate system and the unlabeled products
analyzed by SDS-polyacrylamide gel electrophoresis
(SDS-PAGE).  The two RNA's program the synthesis of

25   distinct translation products whose migration
differences are consistent with the predicted
polypeptide lengths of the two forms (Fig. I-4(B),
lanes 2, 3).  Cytoplasmic extracts from untreated IM-9
cells and IM-9 cells treated with 1 microM triamcino-

30   lone acetonide serve as markers (Fig. I-4(B), lanes
4,5) for the 94K receptor (the 79K form represents a
putative receptor degradation product) (Wrange, et al.,
1984). Note that after steroid treatment, the intensity
of the 94K band is reduced, corresponding to tighter

35   receptor/chromatin binding and, therefore, receptor
translocation to the nucleus.  The alpha form

co-migrates with the 94K band of the negative receptor
while the beta form migrates more rapidly (see Fig.
I-4(B), compare lanes 2,3 with lanes 4,5).  A

5    comparison of cytoplasmic extracts from various human
and mouse cell lines reveals the presence of only the
alpha-receptor (see Fig. I-4(B), lanes 6-9).
Interestingly, the mouse ADR6 lymphoma cell line
(Danielsen, et al., 1984), selected for resistance to

10   steroid-induced lysis, contains no steroid-binding
activity and shows no immunoreactive receptor (see Fig.
I-4(B), lane 7).  Therefore, based on characterization
of multiple receptor cDNA clones and receptor protein
by immunoblot analysis, we conclude that the

15   predominant physiological form of the glucocorticoid
receptor is the alpha (94K) species.

(d) EXPRESSION OF hGR IN VITRO

    To provide additional evidence that the cloned
receptor is functional, we investigated the possibility

20   that the in vitro-translated products might be able to
selectively bind corticosteroids.  Accordingly, the
rabbit reticulocyte lysate was incubated with the
radiolabeled synthetic glucocorticoid analogue
$^3$H-triamcinolone acetonide ($^3$H-TA) before or after
addition of in vitro-synthesized alpha or beta hGR RNA.

25   As shown in Fig. I-5, those lysates programmed with
alpha-hGR RNA acquired selective steroid-binding
capacity; unexpectedly, the beta-receptor synthesized
in vitro failed to bind competable $^3$H-TA.  The in
vitro-synthesized alpha-hGR bound radiolabeled steroid

30   which could be competed with by addition of excess
unlabeled cortisol or dexamethasone; however, binding
of $^3$H-TA was not effectively competed with by addition
of excess unlabeled oestrogen or testosterone.  In
contrast, excess progesterone constituted an effective

35   competitor, consistent with the previously reported
anti-glucocorticoid activities of progesterone

(Rousseau, et al., 1972). To confirm these results, the competition experiments were repeated with native glucocorticoid receptor prepared from extracts of human lymphoid cells. Both the in vitro-translated receptor

5 and the natural in vivo receptor have nearly identical properties with regard to steroid binding and competition with excess unlabeled steroid analogue (see Fig. I-5).

(e) hGR SEQUENCES MAP TO AT LEAST TWO GENES

10        The human glucocorticoid receptor gene has been functionally mapped to chromosome 5. Analysis of somatic cell hybrids constructed by fusing receptor-deficient mouse T cells (EL4) with human receptor-containing T cells (CE.-C7) indicated that

15 segregants expressing the wild-type CEM-C7 receptor maintained human chromosome 5 while dexamethasone-resistant segregants had lost this chromosome (Gehring, et al., 1985).

       To confirm the authenticity of our cDNA

20 clones, we mapped receptor cDNA sequences using Chinese hamster/human somatic cell hybrids containing only human chromosome 5 (HHW454). DNA's extracted from human placenta, HHW454 hybrid cells and Chinese hamster ovary (CHO) cells were digested with EcoRI or HindIII

25 restriction endonucleases and separated on a 0.8% agarose gel. DNA fragments transferred to nitrocellulose were probed with a portion of the receptor-coding region derived from nucleotides 570-1,640 (See hGR1.2 in Fig. I-1(A)). In addition to

30 CHO-specific EcoRI bands of 6.8 and 17 kbp, DNA from the hybrid cell line also contains human-specific bands of 3.0 and 5.0 kbp (see Fig. 6a, lanes 2, 3 of Hollenberg, et al., 1985). (The study disclosed herein as Experimental Section I was published as Hollenberg,

35 et al., 1985. Figures 6 and 7 appear in the paper but not in the present specification.) Unexpectedly, a DNA

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fragment of 9.5 kbp is found in otal human DNA but not in the hybrid line (see Hollenberg, et al., 1985, Fig. 6a, lane 1). Similarly, HindIII digestion revealed a

5  7.5 bkp band that is not present in the chromosome 5 hybrid cell DNA (see Hollenberg, et al., 1985, Fig. 6a, lane 4). These results indicate that the receptor cDNA maps to human chromosome 5, but that there are additional receptor-related sequences elsewhere in the

10  genome. To map these sequences, we used a dual-laser fluorescence-activated cell sorter (FACS) to sort mitotic chromosome suspensions stained with DIPI/chromomycin in conjunction with Hoechst 33258 chromomycin; this technique allows separation of the 24

15  human chromosome types into 22 fractions (Lebo, et al., 1984). After the chromosomes were sorted directly onto nitrocellulose, the chromosomal DNA was denatured and hybridized to the hGR cDNA probe. In addition to confirming the chromosome 5 localization, additional

20  sequences were found on chromosome 16 (see Hollenberg, et al., 1985, Fig. 6b). To confirm this localization, DNA's from mouse erythroleukaemia cells and a mouse erythroleukaemia cell line containing human chromosome 16 (see Bode, et al., 1981) were digested with HindIII

25  and probed with hGR cDNA (see Hollenberg, et al., 1985, Fig. 6c); as predicted, the only DNA fragment found in the hybrid and not in the control was the 7.5-kbp DNA fragment, thus establishing the chromosome 16 assignment (see Hollenberg, et al., Fig. 6c, lanes 1,

30  2).

Additional Southern blot analyses using the EcoRI-XbaI fragments from OB7 and OB10 3'-untranslated regions revealed hybridization only to chromosome 5 (data not shown). We conclude that both the alpha- and

35  beta-receptor cDNA's are probably encoded by a single gene on chromosome 5 and suggest that the two cDNA forms are generated by alternative splicing. In

addition, we conclude that another gene residing on human chromosome 16 contains homology to the glucocorticoid receptor gene, at least between

5  nucleotides 570 and 1,640. It is not clear whether these sequences on chromosome 16 represent a related steroid receptor gene, a processed gene or pseudogene, or a gene that shares a common domain with the gene for the glucocorticoid receptor. Genomic cloning and DNA

10 sequencing may provide the answer.

To determine the size of the mRNA encoding the glucorcorticoid receptor, Northern blot hybridization (Bode, et al., 1981) experiments were performed using cytoplasmic mRNA isolated from a human fibroblast cell

15 line, HT1080. Using the hGR1.2 coding sequence as probe, multiple mRNA's of 5.6, 6.1 and 7.1 kb were detected. Treatment of these cells with glucocorticoids for 24 h leads to a 2-3-fold reduction in receptor mRNA's, suggesting a potential negative

20 feedback regulation.

## I. D. DISCUSSION

Structural analysis of the glucocorticoid receptor is a prerequisite for gaining insight into the mechanisms by which this regulatory molecule exerts its

25 effects on gene transcription. Here, we have presented the primary sequence of the human glucocorticoid receptor deduced from nucleotide sequence analysis of cDNA clones.

Isolation of hGR cDNA's has revealed the

30 existence of multiple mRNA's encoding at least two forms of the polypeptide. The predicted proteins differ at their carboxy termini by the substitution of 50 amino acids in the case of alpha-hGR and 15 amino acids in the case of beta-hGR. The alpha

35 glucocorticoid receptor is the major form identified in several human cell lines and cDNA libraries. However, a recent report by Northrop, et al. (1985)

characterizes two forms of the receptor in mouse
lymphoid cells.  The relationship of alpha- and
beta-hGR to the mouse doublet species remains to be
established.  Also, the cellular distribution and

5    potential function of beta-hGR are unclear, although it
is possible that variant receptors are used for
tissue-specific functions.  We are now generating
antisera to synthetic peptides specific for each human
receptor form to elucidate their tissue-specific

10   expression.

        Among the cDNA's selected using the
immunopositive phage DNA insert hGR1.2A as probe were
those containing 3' ends similar to OB7, except that
polyadenylation was signaled earlier by the use of an

15   AATAAA at nucleotide 3,101.  These clones have been
isolated from both human fibroblast and placental
libraries (data not shown).  Alternative poly(A) site
selection is a feature of many eukaryotic transcription
units (Darnell, 1982).  In some instances, selection of

20   poly(A) sites specifies particular polypeptide products
(Amara, et al., 1982; Rosenfeld, et al., 1983; Alt, et
al., 1980; Schwarzbauer, et al., 1983) while in other
cases, alternative poly(A) site selection produces no
change in the primary structure of the polypeptide

25   (Setzer, et al., 1982) while in other cases,
alternative poly(A) site selection produces no change
in the primary structure of the polypeptide (Setzer, et
al., 1982).  The selection of poly(A) sites during
receptor transcription may (1) alter the stability of

30   the mRNA in a particular tissue, (2) lead to splicing
changes, or (3) be random, with no physiological
consequence.

        The *in vitro* translation studies described
here provide direct evidence that the cloned molecule

35   encodes the complete glucocorticoid receptor.  First,
the *in vitro*-translated product is identical in size to

the native glucocorticoid receptor and is
immunologically reactive with receptor-specific
antiserum.    Second, the in vitro-translated protein
acts functionally as a glucocorticoid receptor in that
5     it is capable of selectively binding the synthetic
glucocorticoid triamcinolone acetonide.    This binding
is specifically competed with by glucocorticoids,
glucocorticoid analogues and progesterone but is not
competed with by the sex steroids testosterone and
10    oestrogen.    In this respect, the in vitro-translated
receptor behaves identically to the in vivo receptor
from human lymphoid cells, providing the first evidence
of a function for the cloned molecule.    The acquisition
of steroid-binding properties does not appear to
15    require any specific modifications or, if it does,
these modifications can occur in the in vitro
translation mix.

The results presented here provide the
information necessary for studying the molecular
20    interactions of a eukaryotic transcriptional regulatory
protein with its target genes.    These structural
studies provide a basis from which the glucocorticoid
receptor, its gene, and its RNA products can be
analyzed.    Furthermore, the ability to express receptor
25    in vitro provides a novel means by which the
consequence of specific in vitro mutagenesis can be
rapidly tested.    Finally, the isolation of genes
responsive to glucocorticoids and specific regulatory
elements by both mutagenic and protein-binding studies
30    suggests that this protein can serve as a very useful
model for analysis of inducible eukaryotic gene
regulation.

I. E. DETAILED DESCRIPTION OF FIGURES REFERRED

TO IN EXPERIMENTAL SECTION V

35    FIGURE I-1 (A) and (B).

Human glucocorticoid receptor cDNA sequencing

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strategy and schematic representation of cDNA clones.
A, the composite cDNA for the alpha glucocorticoid
receptor is represented at the top, with noncoding
(lines) and coding (stippled portion) sequences

5  indicated.  Common 6-nucleotide restriction enzyme
sites are shown.  Overlapping cDNA inserts used to
determine the sequence are shown:  arrows beneath the
regions sequenced show the direction and extent of
sequencing.  The dashed line at the 3' end of OB10

10  indicates divergent sequence.  Numbers refer to
nucleotide positions in OB10 relative to the 5'-most
transcribed sequence.  B, cDNA's encoding the alpha and
beta forms of the receptor (OB7 and OB10,
respectively).  The 5' end of OB7 (broken lines) is

15  contributed by the OB10 clone.  Protein-coding
information is represented by wide bars; untranslated
sequences are indicated by thin bars.  Nucleotides and
amino acids are numbered above and below the coding
sequence, respectively.  Common DNA sequences extend to

20  nucleotide 2,313 (amino-acid residue 727), at which
point the alpha- and beta-receptor forms diverge, with
the alpha cDNA's (OB12, OB7) continuing in an open
reading frame for 150 nucleotides (50 amino acids) and
the beta cDNA (OB10) continuing for 45 nucleotides (15

25  amino acids; see Fig. I-3(B)).  Hexanucleotide signals
(AATAAA) just upstream of the poly(A) in the clones are
indicated, with the first hexanucleotide in OB7 serving
as poly(A) in OB12.

FIGURE I-1 (A) and (B) METHODS

30        The inserts hGR1.2, hGR2.9 and hGR5.16 were
isolated from a lambda gt11 IM-9 lymphoid cell cDNA
library as described previously (Weinberger, et al.,
1985).  Two clones were isolated from cDNA libraries
constructed by H. Okayama in pcD (Okayama, et al.,

35  1983) using poly(A)+ mRNA from GM637 human fibroblasts
(OB7) and primary human fibroblasts (OB10).  Screening

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was performed with the hGR1.2-cDNA, radiolabeled by
nick-translation with 32P-dCTP. Sequences were
determined by the chemical cleavage method of Maxam and
Gilbert (1977).

5    FIGURE I-2 (1) and (2).

      cDNA and predicted protein sequence of human
glucocorticoid receptor. The complete alpha coding
sequence and OB7 3'-untranslated region are shown, with
the deduced amino acids given above the long open

10   reading frame. An upstream in-frame stop codon at
nucleotides 121-123 and putative additional
polyadenylation signals in OB7 are underlined.

FIGURE I-3 (A) and (B).

      Restriction map and nucleotide sequence of the

15   3' end of the human glucocorticoid receptor beta cDNA.
A, The common 6-nucleotide restriction enzyme sites are
shown for the 3'-untranslated region of OB10. B, The
cDNA sequence of the beta form (OB10) from nucleotide
2,281 to 3,820 compared with the protein-coding

20   information found in the 3'-terminal coding portion of
the alpha form (OB7). Amino acids encoded by each of
the cDNA's are presented above the nucleotide
sequences. Putative polyadenylation signals (AATAAA)
in the 3'-untranslated sequence of OB10 are underlined.

25   FIGURE I-4 (A) and (B).

      Immunoblot comparison of hGR translated in
vitro with in vivo hGR from cell extracts. A, The
vectors constructed for in vitro transcription of the
hGR cDNA sequence. The complete alpha (pGR107) and

30   beta (pGR108) coding sequences were placed under the
transcriptional control of the SP6 promoter in pGEM1.
Vector sequences, noncoding cDNA sequences and coding
sequences are indicated by thin lines, thick bars and
boxed regions, respectively. The poly(A) tract of ~60

35   nucleotides is indicated by $A_{pi}$. Divergent coding
sequences are indicated by striped and stippled

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regions.  B, Western blot analysis of _in vitro_
translation products and cell extracts.  Unlabeled
translation products synthesized in a rabbit
reticulocyte lysate system with no added RNA (lane 1)
5      or with RNA synthesized from pGR108 (beta, lane 2) or
pGR107 (alpha, lane 3) were fractionated on a 7.5%
SDS-polyacrylamide gel.  Additional lanes are:
cytoplasmic extracts from IM-9 (lane 4), IM-9 treated
with 1 microM triamcinolone acetonide (lane 5), HeLa
10     (lane 6), ADR6.M1890.AD1 mouse lymphoma (lane 7), S49
mouse lymphoma (lane 8) and EL4 lymphoma (lane 9).
Proteins were transferred to nitrocellulose and probed
with anti-hGR antibody, followed by 125I-labeled
_Staphylococcus_ _aureus_ protein A as described previously
15     (Weinberger, et al., 1985).

FIGURE I-4 (A) and (B) METHODS.

To construct an expression vector containing
the entire alpha coding sequence shown in Figs. I-2(1)
and I-2(2), the 3' coding sequence of OB7 was fused to
20     OB10 5' coding information.  OB7 was partially digested
with EcoRI, completely digested with XbaI, and the
1.20-kbp fragment was gel-purified and ligated with
EcoRI/XbaI-digested OB10 to produce the intermediate
pOB107.  The entire pOB107 cDNA sequence including the
25     5' poly(G) tract (11 nucleotides, nt) and 3' poly(A)
tract (~60 nt) was excised by partial PstI/complete
BamHI digestion.  The resultant 3.5-kb fragment was
gel-purified and inserted between the PstI and BamHI
sites of pGEM1 (Promega Biotec) to yield pGR107.
30     Plasmid pGR108 was directly constructed from pOB10 by
partial PstI/complete BamHI digestion and insertion of
the resulting cDNA insert into the corresponding sties
of pGEM1.  Capped SP6 transcripts were synthesized from
PvuII-linearized pGR107 and pGR108, as described by
35     Krieg and Melton (1984), with simultaneous capping
effected by reduction of the GTP concentration from 400

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to 100 microM and addition of m⁷GppG (Pharmacia) to 500
microM.   Transcripts were purified by P60
chromatography and translated with micrococcal
nuclease-treated rabbit reticulocyte lysate (Promega
5   Biotec) in conditions suggested by the manufacturer.
Preparation of IM-9 cytosol from steroid-treated cells
was as described previously (Weinberger, et al., 1985).
Size markers are phosphorylase B (97K), bovine serum
10  albumin (66K) and ovalbumin (45K).

FIGURE I-5.

Steroid-binding of alpha-hGR translated in
vitro.  Binding to IM-9 cytosol extract (stippled bars)
and to reticulocyte lysate containing SP6-generated
15  alpha-hGR RNA (GR107; open bars) are shown.  Bars
represent bound $^3$H-triamcinolone acetonide (TA)
determined with a 100-fold excess of various steroid
competitors; 100% competition was determined using
unlabeled TA as competitor.  The values represent the
20  mean of triplicate determinations, with error bars
showing P<0.05.  Steroid competitors are dexamethasone
(Dex), cortisol (Cort), progesterone (Prog),
testosterone (Test), and oestradiol (Oest).

FIGURE I-5 METHODS.

25  Binding assays were performed in 100
microliters containing 10 mM Tris-HCl pH 7.4, 100 mM
NaCl, 1 mM EDTA, 10 mM sodium molybdate, 10
dithiothreitol, 150 mM $^3$H-TA (20 Ci mmol$^{-1}$; Amersham)
and 10 microliters translation mixture or 100 microgram
30  fresh IM-9 cytosol.  Unlabeled steroid competitor (15
microM) was added as indicated.  After 2 h at 0°C,
samples were extracted twice for 5 min. each with 5
microliter of 50% dextran-coated charcoal to remove
unbound steroid, and counted.  Uncompeted and fully
35  competed values for the alpha glucocorticoid receptor
(GR107) were 490 and 290 c.p.m., respectively.
Reticulocyte lysate translation mixtures without added

transcript or programmed with beta-receptor SP6 RNA
(GR108) contained no competable $^3$H-TA binding.

ADDITIONAL FIGURES.

5       The scientific study presented here as
Experimental Section I was published in Nature,
318:635-641 (1985). The Nature publication contain two
Figures which are not included in this Experimental
section. Those figures are: Figure 6, Chromosome
10 mapping analysis of hGR cDNA; Figure 7, Northern blot
analysis of hGR cDNA.

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EXPERIMENTAL SECTION II
FUNCTIONAL DOMAINS OF THE
HUMAN GLUCOCORTICOID RECEPTOR
II. A. SUMMARY

5       Human glucocorticoid receptor (hGR) produced
in CV-1 cells via transfection of an hGR expression
vector functions as a necessary and sufficient factor
for the transcriptional activation of the MTV-CAT
10  fusion gene.  The magnitude of the induction (500-1000
fold) reveals that the hGR may act as a transcriptional
"switch", converting a silent promoter containing a
glucocorticoid response element to an activated state.
Stimulation of transcription of the MTV-CAT gene fusion
15  by hGR is not dependent on transcriptional factors that
are limiting in CV-1 cells.  Characterization of 27
insertional mutants of the hGR allowed the location of
at least four functional domains, two of which
correspond to the predicted DNA- and steroid-binding
20  domains.  The other two domains are referred to as tau
for their potent effects on transcription.  This raises
the possibility that other regions in the receptor are
necessary for full transcriptional activation but are
not specifically involved in steroid or DNA binding.

25                II. B. INTRODUCTION
        The primary structure of two classes of
steroid hormone receptors have been elucidated by
cloning and sequencing of their cDNA.  As Experimental
Section I discloses, identification of cDNA's encoding
30  the human glucocorticoid receptor (hGR) revealed two
forms of the protein, of 777 (alpha) and 742 (beta)
amino acids, which differ at their carboxyl termini.
(The Experimental Section I disclosure has been
published as Hollenberg, et al., 1985.)  The human
35  estrogen receptor is a somewhat smaller protein of 595
amino acids (Greene, et al., 1986; Green, et al.,
1986).  Amino acid sequence comparisons revealed

extensive regions of homology not only between the two
classes of receptors but also with the v-erb-A oncogene
product of avian erythroblastosis virus (Weinberger, et
al., 1985; Greene, et al., 1986; Green, et al., 1986).
5   This supports the suggestion that steroid receptor
genes and the c-erb-A proto-oncogene are derived from a
common primordial ancestral regulatory gene
(Weinberger, et al., 1985).

10         On the basis of the amino acid sequence of hGR
deduced from the cloned cDNA (see Experimental Section
I), the locations of functionally and immunologically
important regions of the protein have been proposed
(Weinberger, et al., 1985).  These include an
15   immunological domain located in the amino-terminal half
of the protein, a DNA-binding domain that shows
structural similarities with other DNA-binding
proteins, and the glucocorticoid-binding site localized
near the carboxyl terminus of the molecule.  However,
20   the location of each domain is tentative, and no domain
involved in the activation of transcription itself has
been identified.  In this study, we sought to confirm
the proposed sites of the functional domains within the
hGR and to find other regions of importance by
25   introducing amino acid alterations in the hGR protein.
We first developed a novel expression system in monkey
kidney cells in which the synthesis of functional hGR
is directed by the transcription of the cDNA under the
control of the long terminal repeat (LTR) of the Rous
30   sarcoma virus (RSV).  The functions of the synthesized
receptor were monitored by the induction of
transcription of the mouse mammary tumor virus (MTV)
LTR as measured by chloramphenicol acetyltransferase
(CAT) assays (Gorman, et al., 1982a) and steroid
35   hormone binding.  This new expression system allowed us
to investigate the effect of insertional mutagenesis on
the various functions of the receptor, which led us to

propose a more detailed model of the domain structure
of the hGR.  Our results, based on the analysis of 27
insertional mutations, confirm the notion that the
5   glucocorticoid receptor is composed of discrete
functional domains (Weinberger, et al., 1985).  In
addition, they identify additional sequences outside
the proposed DNA- and steroid-binding domains, which,
as stated above, we refer to as tau for their potent
10   effects on transcription.

## II. C. RESULTS

### (a) ASSAY SYSTEM AND EXPERIMENTAL DESIGN

The assay system and strategy used to study
the expression of functional hGR from the cloned cDNA
15   (See Experimental Section I) is shown in Figure II-1.
In these experiments, a glucocorticoid-responsive
promoter/enhancer element linked to a reporter gene was
introduced into a receptor-negative cell.  Thus, in
principle, this construction should be
20   transcriptionally inactive.  For our assay, we chose to
use the MTV LTR fused to the sequence coding for
chloramphenicol acetyltransferase (CAT) (EC2.3.1.28).
It has been demonstrated previously that glucocorticoid
hormones stimulate the rate of transcription of MTV DNA
25   (Ringold, et al., 1977) by increasing the efficiency of
transcription initiation at a unique site within the
MTV LTR (Ucker, et al., 1983).  Moreover,
glucocorticoid receptors bind specifically to DNA
sequences mapped within the MTV LTR (Payvar, et al.,
30   1983), which can confer glucocorticoid responsiveness
to a heterologous promoter (Chandler, et al., 1983).
Cotransfection of pMTVCAT (or pGMCAT) with a receptor
expression plasmid provides functional receptors that
allow induction of CAT activity upon treatment of the
35   transfected cells with glucocorticoid hormone.  In
addition, biochemical studies such as steroid binding
activity and Western blot analysis of the expressed

receptors can be performed simultaneously.

The expression vector linking the RSV LTR to full length hGR cDNA (pRShGR alpha) was designed to
5  obtain high levels of expression in a wide range of host cell types.  The vector pRShGR alpha is a derivative of pRSVCAT (Gorman, et al., 1982b) in which the coding sequence of the CAT gene was replaced by the hGR cDNA.  The origin of replication of SV40 was
10  introduced into the vector to allow the recombinant plasmid to propagate to high copy numbers in COS-1 (referred to as COS) monkey kidney cells that express T antigen (Tag) (Gluzman, 1981).  COS cells and parental cell line CV-1 offer the additional advantage of having
15  undetectable levels of glucocorticoid receptors (unpublished observation and Figures II-2 and II-3).

(b) EXPRESSION OF FUNCTIONAL hGR

The above assay was designed to overcome some of the major difficulties encountered in studying the
20  mechanisms of action of steroid hormone receptors. These difficulties include low intracellular levels of receptor, possible heterogeneity of receptors, and lack of a quantifiable bioassay system to test receptor functions.  Accordingly, we first looked at the
25  relative amount of hGR that could be made by COS cells transfected with pRShGR alpha.  Figure II-2 (right lane), a Western blot analysis of transfected COS cells, demonstrates that COS cells synthesized an hGR polypeptide of 94 kd that is indistinguishable with
30  respect to mobility from the hGR present in the IM9 cell line (left lane).  Moreover, the amount of hGR present in transiently transfected COS cells is greater than the level found in IM9 cells, which contain between 100,000-200,000 receptors per cell (Harmon, et
35  al., 1984).  This expression system not only provides us with cells carrying high intracellular levels of hGR, but eliminates the possibility of receptor

microheterogeneity, which could interfere in the
functional study of hGR.

5    To test the functional capability of the
expressed hGR as a positive transcriptional factor, we
performed CAT assays with cell extracts obtained after
cotransfection with pMTVCAT and pRShGR alpha.
Transfection into both COS and the parental CV-1 cells
was examined. As expected (Alwine, 1985), the presence
10   of SV40 Tag in COS cells increased basal activity of
the MTV LTR (data not shown). Thus, CV-1 cells, which
do not express the SV40 Tag, were used to achieve
maximal induction. As shown in Figure II-3,
cotransfection of pMTVCAT with a control plasmid does
15   not generate CAT activity in CV-1 cells. Similarly,
cotransfection of pMTVCAT and pRShGR alpha does not
produce any CAT activity. However, treatment of the
same cotransfected CV-1 cells with dexamethasone (DEX)
turns on the transcription of the MTV-CAT fusion gene.
20   The induction factor is very large (approximately
500-1000 fold) since basal levels of CAT activity
produced by pMTVCAT are barely detectible (often zero)
in CV-1 cells. As a control experiment, we
cotransfected the beta form of the hGR (see
25   Experimental Section I), which was shown to be unable
to bind steroids (see Table II-1). Figure II-3
demonstrates that hGR beta is not functional in our
expression assay. The activation of transcription by
hGR is also restricted to promoters containing a
30   glucocorticoid-responsive element. When pMTVCAT was
substituted for pMTIaCAT, a plasmid containing the
regulatory region of the human metallothionein Ia gene,
which is responsive to heavy metals but not to
glucocorticoids, no induction of CAT activity was
35   observed after hormonal treatment of the transfected
CV-1 (data not shown). These results demonstrate that
in cells the hGR acts as a necessary and sufficient

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factor that functions as a steroid-dependent
transcriptional switch.

Based on this assay, the activation of the
receptor by steroids could be examined. As shown in
Figure II-4(A), DEX exhibits an $ED_{50}$ value of 3 nM on
hGR-induced CAT activity, which is in agreement with
$ED_{50}$ values observed for DEX (5 nM) in a variety of
psychological actions. Specificity of hGR action was
further tested by treating cotransfected CV-1 cells
with 100 nM testosterone, oestradiol, and progesterone.
These steroids failed to induce CAT activity with the
exception of progesterone, which stimulated hGR
function at a value of 1% of the maximal induction
produced by DEX (data not shown). These results
indicate that transfected CV-1 cells synthesize
functional hGR that interacts with pharmacological
ligands with the specificity and concentrations of the
natural receptor.

Studies on specific interaction between
enhancer-containing molecules and cellular components
have shown that CV-1 cells contain limiting amounts of
cellular factors required for the function of certain
viral enhancers (Scholer and Gruss, 1984). In those
cells, CAT activity generated by transfected pSV2CAT
(Gorman, et al., 1982a) reaches a plateau at 0.3 pmol
of plasmid per dish. Similarly, if the hGR interacts
with limiting factors, we should be able to saturate
the CAT activity induced by transfection of increasing
amounts of pRShGR alpha with a constant quantity of
pMTVCAT. In this experiment, 2 pmol (5 micrograms) of
pMTVCAT DNA was used and increasing amounts of pRShGR
alpha DNA were added, together with nonspecific carrier
DNA, to yield a total of 30 micrograms per dish. Figure
II-4(B) demonstrates that CAT activity could be
detected when as little as 0.03 pmol (100 ng) of pRShGR
alpha per dish was transfected and that no plateau in

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CAT activity was reached.  These data suggest that
stimulation of transcription of the MTV-CAT fusion gene
by hGR is not dependent on transcriptional factors that
are limiting in CV-1 cells.

5  (c) MAPPING OF FUNCTIONAL DOMAINS IN hGR

          Understanding the mechanisms by which hGR
regulates gene transcription first required the
characterization of its functional domains.  Based on
limited proteolysis studies of the glucocorticoid
10 receptor (Carlsdedt-Duke, 1982; Dellweg, et al., 1982;
Wrange, et al., 1984; Reichman, et al., 1984) and on
the analysis of the primary structure of the hGR (see
Experimental Section I), a model for the structure of
the receptor has been proposed (Weinberger, et al.,
15 1985).  This model identifies three major domains - an
immunological domain spanning from amino acid 145 to
280, a DNA-binding domain extending from amino acids
421 to 481, and a steroid-binding domain located near
the carboxyl terminus of the protein.  To test this
20 model, we generated 27 site-specific insertional
mutations in the glucocorticoid receptor coding
sequence via a linker-scanning approach.  These
genetically engineered mutants were then assayed for
their ability to stimulate gene transcription and to
25 bind steroid hormone.

          To generate linker-insertion mutants of the
hGR, the plasmid pRShGR alpha was first linearized by
partial cleavage using a restriction enzyme that
cleaves DNA molecules with high frequency.  The linear
30 form of the plasmid was isolated and a BamHI linker was
added to restore the open reading frame encoding the
hGR.  The resulting mutants carry three or four
additional amino acids, which disrupt the wild-type
sequence of the protein.  Using this technique, we have
35 generated a random series of hGR mutants (Figure II-5).
The ability of these mutants to express full-length hGR

was estimated by Western blot analysis. The amounts of
hGR produced were shown not to vary by more than 30%,
and thus none of the mutants appear to destabilize the
expressed protein.

5

The functional properties of each mutant are
compared with that of the wild-type hGR in Table II-1.
CAT activity induced by 12 out of 27 hGR mutants was
comparable with wild-type level. Analysis of the 15
hGR mutants having a diminished or a complete loss of
10 function, as assayed by induction of CAT activity,
shows that they belong to four separate groups. A
cluster of these mutants located between amino acids
120 and 215 in the so-called immunogenic domain forms
the first group. Although no specific function has
15 been assigned to this region of the receptor molecule,
three mutants (I120, I204, and I214) show decreased
capacity to induce CAT activity. Those mutants
retained their full ability to bind steroids.

Perhaps not surprisingly, the second group of
20 defective mutants is found in the putaive DNA-binding
domain of the receptor. This domain is cysteine-rich
and consists of two repeat units of about 25 amino
acids each, which could fold into a loop structure
25 coordinated by a $Zn^{2+}$ ligand (Miller, et al., 1985). In
mutant I422, the sequence motif $Cys-X_2-Cys$ is changed
to $Cys-X_5-Cys$. The presence of the additional amino
acids completely abolishes receptor function. Mutant
I440 bears a similar insertion of four amino acids
30 between the two other cysteines involved in the
formation of the first loop and also fails to induce
any detectable level of CAT activity. On the other
hand, mutant I428 extends the length of the loop
itself, from 13 to 17 amino acids. Although severely
35 diminished, induction of CAT activity by I428 is still
measurable. Steroid-binding capacity of all three
mutants located in the DNA-binding domain was shown to

be in the range of wild-type level.  The third region
affected by the mutations is located next to the
DNA-binding domain.  Mutants I488 and I490 show low
5  levels of CAT activity but bind steroid efficiently.
The fourth group covers the last 200 amino acids of the
receptor protein.  Five mutants (I582, I589, I599,
I626, and I696) show undetectable levels of CAT
activity.  This lack of functional activity is
10  correlated with their total incapacity to bind
dexamethasone.  These results show that the
steroid-binding region encompasses a large portion of
the protein, all clustered near the C terminus.  In
contrast to the amino terminus of the molecule, this
15  region is extremely sensitive to changes in the primary
structure of the receptor.

(d) TABLE II-I

Functional Properties of hGR Mutants

| hGR | Inserted Amino Acids | CAT Activity (%) | DEX Binding (%) |
|---|---|---|---|
| alpha | - | 100 | 100 |
| I9 | RIR | 117 | NT |
| I37 | RIRA | 95 | NT |
| I102 | GSV | 130 | NT |
| I120 | RGSA | 2 | 76 |
| I204 | RIR | 3 | 125 |
| I214 | RGSA | 2 | 79 |
| I262 | ADPR | 97 | NT |
| I289 | RIR | 125 | NT |
| I305 | ADPR | 86 | NT |
| I346 | ADPR | 19 | 107 |
| I384 | RIR | 101 | NT |
| I403 | ADPR | 114 | NT |
| I408 | ADPR | 55 | NT |
| I422 | GSV | 0 | 105 |
| I428 | RIRA | 2 | 92 |
| I440 | ADPR | 0 | 69 |

| | | | |
|---|---|---|---|
| I488 | GSV | 15 | 96 |
| I499 | RIRA | 10 | 115 |
| I515 | RIR | − 109 | NT |
| I532 | GSV | 115 | NT |
| I550 | ADPR | 5 | 19 |
| I582 | RIR | 0 | 0 |
| I589 | GSV | 0 | 0 |
| I599 | SDP | 0 | 0 |
| I626 | ADPR | 0 | 0 |
| I684 | RGSA | 79 | 81 |
| I696 | RGSA | 0 | 0 |
| beta | C-terminal deletion | 0 | 0 |

CV-1 or COS cells were transfected with pRShGR alpha,
pRShGR beta, or a mutated hGR alpha and assayed for CAT
activity and steroid-binding capacity. After
transfection, CV-1 cells were cultured for 2 days in
the presence of 10 nM DEX before cell lysis and CAT
assay; COS cells were maintained in normal media. The
two parameters are quantified as percentage (%) of
wild-type hGR activity. Amino acids inserted in hGR
alpha are given in the one-letter code. NT means not
tested. Differences in amino acid composition between
hGR alpha and hGR beta are represented in Figure II-5
and in Experimental Section I.

## II. D. DISCUSSION

We have shown that hGR produced in CV-1 cells
via transfection of an hGR expression vector functions
as a necessary and sufficient factor for the
transcriptional activation of the MTR-CAT fusion gene.
The magnitude of the induction reveals the hGR may act
as a transcriptional "switch", which can convert a
silent promoter containing a glucocorticoid response
element to an activated state. Unlike other
transcriptional factors that are constitutively active,
stimulation of transcription by hGR is totally

dependent upon the presence of glucocorticoid hormones (Figures II-3 and II-4(A)).  The production of an excessive quantity of the protein wit..in a cell is not sufficient to induce transcription of a regulated gene.

5     The mechanism by which hGR is activated by the hormone is poorly understood  but, in analogy with the cyclic AMP-binding protein, is likely to involve allosteric transitions within the protein (McKay and Steitz, 1981; Gages and Adhya, 1985).

10          We have observed that activation of transcription by hGR is not restricted by factors present in limiting quantity in CV-1 cells (Figure II-4(B)).  These results suggest that the binding of hGR-steroid complex to a glucocorticoid-responsive

15    enhancer is sufficient to increase the activity of general transcriptional factors at nearby promoters. Similar properties for several other transcriptional factors have been reported.  For example, Adf1, a transcription factor that activates the proximal

20    promoter of the alcohol dehydrogenase (Adh) gene in D. melanogaster, binds the Adh template DNA in the absence of other protein factors and requires only endogenous RNA polymerase II and a fraction containing another general transcription factor to activate initiation of

25    Adh RNA synthesis (Beberlein, et al., 1985).  In a different type of experiment using the recombinant plasmid pSV2CAT, which contains SV40 enhancer/promoter elements, Scholer and Gruss (1984) have shown a requirement of a cellular molecule(s) for the function

30    of enhancer-containing DNA.  Their experiments indicated the presence of a limited amount of cellular factor(s) required for the activation of the CAT gene by the SV40 enhancer element.  However, no exhaustion of general transcriptional factors was observed.  These

35    data suggest that the mechanism of action of specific positive transcriptional factors is likely to involve

alterations in chromatin structure induced by the
factor itself that would facilitate the activity of
general transcription factors or the polymerase itself
(Moreau, et al., 1981; Wasylyk, et al., 1983).  It has
5   been previously shown that glucocorticoid treatment
causes both reversible and persistent changes in
chromatin structure in DNA regions containing a segment
of the MTV LTR (Zaret and Yamamoto, 1984).  The
mechanism by which bound receptors protentiate promoter
10  activity remains to be completely elucidated.  However,
the availability of a system that overexpresses hGR
will facilitate the future studies on the molecular
basis of transcription activation by positive
transcriptional factors.
15
        The results of the characterization of the 27
insertional mutants supports and extends our previous
suggestion that the human glucocorticoid receptor is
composed of a series of functional domains.  It is
20  noteworthy that all mutants that affect steroid binding
are clustered at the carboxyl terminus.  In addition to
suggesting that this region functions as a discrete
domain encoding hormone specificity, the results imply
the possibility that the other domains identified
25  within the receptor may serve discrete functions.
Accordingly, the ability of the receptor to recognize
and to interact with specific DNA sequences appears to
reside in the Cys-Lys-Arg-rich region, which is highly
conserved with the estrogen receptor and the oncogene
30  product v-erb-A.  It seems logical that mutations in
these regions would diminish the ability of the
receptor molecule to activate transcription since
activation depends on both the ability of the ligand to
induce an allosteric transformation and the ability of
35  the transformed molecule to recognize and interact with
the DNA.  Based on the initial model of steroid
receptor structure (Weinberger, et al., 1985), these

were expected outcomes of a mutagenic characterization.
The unexpected outcome, however, is the identification
of at least two other regions influencing

5  transcriptional activity.  This raises the intriguing
possibility that other domains are present in the
receptor that are necessary for transcriptional
activation but are not specifically involved in either
steroid or DNA binding.  Mutants I120, I204, and I214

10  bind steroid with wild-type affinity but have
diminished transcriptional activity.  These mutants
clearly demonstrate that this domain, which we refer to
as $tau_1$, is functionally important and required to
obtain complete activity of the hGR.  Interestingly,

15  nonfunctional truncated mutants (i.e., 40 kd) found in
several lines of glucocorticoid-resistant cells are
retained in nuclei more efficiently than the wild-type
receptor, but fail to activate transcription (Yamamoto,
et al., 1976; Andreasen and Gehring, 1981; Westphal, et

20  al., 1984).  The receptor fragment missing in these
"increased nuclear transfer" ($nt^1$) mutants is evidently
the amino terminus of the protein since they retain
hormone-binding capacity.  We note that $tau_1$, coincides
with the major immunogenic domain of the hGR

25  (Weinberger, et al., 1985), indicating that it is
probably on the external surface of the molecule.
Speculations on how this domain can fulfill its
functions include self-interaction leading to receptor
dimerization, possible interactions with general

30  transcriptional factors such as RNA polymerase II,
and/or modulation of DNA binding by exerting allosteric
influence over the remainder of the activated receptor
(Dellweg, et al., 1982).  $Tau_1$ is englobed by the amino
terminus of the receptor, a region which is not held in

35  common with the smaller estrogen receptor.  Perhaps the
estrogen receptor gains the equivalent function of this
domain by interacting with a second protein, or,

alternatively, tau1 may interact with other residues
within the glucocorticoid receptor itself, as opposed
to interacting with other regulatory molecules. The

5    other tau region (which we refer to as tau2) that
affects transcriptional activation is a region that is
present in the estrogen receptor and the v-erb-A
oncogene. Its location also suggests that it may act
as a "hinge" region linking the steroid- and

10   DNA-binding domains. Thus, these mutants could block
the allosteric transformation necessary for receptor
activation.

A third region affected by amino acid
insertions is located in the putative DNA-binding

15   domain described by Weinberger, et al. (1985). This
domain is composed of two repeated units containing a
Cys-Lys-Arg-rich sequence and is the most intensively
conserved when compared with the v-erb-A oncogene and
the estrogen receptor (Weinberger, et al., 1985; Green,

20   et al., 1986; Greene, et al., 1986). These repeated
units were first observed in the factor TF-IIIa
(Miller, et al., 1985) and have since been found by
sequence homology searches in a number of other nucleic
acid-binding proteins (Berg, 1986). Based on

25   experimental and theoretical studies of the factor
TFIIIa, Miller and colleagues (1985) proposed a novel
mechanism by which proteins bind DNA molecules. In
their model, each unit is folded into a "fingered"
structure centered on a zinc ion. A finger could bind

30   to a half-turn of DNA. Mutants I422 and I440 carry an
amino acid insertion that disrupts the motif $Cys-X_2-Cys$
central to the finger model. These mutants are totally
inactive with respect to transcriptional activation but
retain their ability to bind glucocorticoid hormone.

35   Preliminary experiments revealed that these mutants
also fail to translocate to the nucleus after hormone
treatment and to bind DNA in vitro (S. Hollenberg,

unpublished observations). These mutated receptors
demonstrate the functional importance of the finger
motifs present in hGR. The third mutant located in

5  this region, I428, has the finger extended by the
addition of four amino acids. Transcriptional activity
of I428 is greatly impaired (2% of wild-type level),
but is still detectable. Thus, the loops are
apparently more tolerant of change than the

10  zinc-binding motif. The demonstration that finger-like
domains are functionally important in hGR leads us to
propose that steroid hormone receptors are
metallo-proteins, which may have evolved from a
primordial ancestral DNA-binding protein.

15        Together, these data suggest that the receptor
is composed of a melange of regulatory domains, which
may have been pirated over evolutionary time to
condense into the primordial steroid hormone receptor,
which in turn gave rise to the large family of hormone

20  response genes present in the mammalian genome. The
transcriptional activity of this molecule demonstrates
its potential ability to act as a genetic switch, which
is consistent with the role of steroid hormones in
activating a variety of developmental lineages and

25  homeostatic functions. The design of the mutations
allows for the convenient generation of any desired set
of small or large deletional mutants and the ability to
switch domains between related molecules to study
function. In conjunction with the rapid and

30  quantitative functional assays described in this
section, it is now possible to direct specific
questions as to the functional nature of the tau, DNA-,
and steroid-binding domains.

35

II. E. DETAILED DESCRIPTION OF FIGURES
REFERRED TO IN EXPERIMENTAL SECTION II

FIGURE II-1.

5    SCHEMATIC REPRESENTATION OF THE hGR FUNCTIONAL ASSAY

In this assay, an expression vector containing
the hGR alpha cDNA or a mutant derived from it is
cotransfected into CV-1 or COS cells with a plasmid
carry    the CAT gene under the control of the MTV LTR.

10   The cells are then cultured in the presence or absence
of hormone.  CV-1 cells were used to monitor induction
of CAT activity, and COS cells were used to measure
steroid-binding capacity and the expression of hGR
protein.

15   FIGURE II-2.

EXPRESSION OF hGR PROTEIN

COS cells were mock-transfected (center lane)
or transfected with plasmid pRShGR alpha (right lane)
and analyzed 2 days later for the presence of hGR

20   protein.  Crude cytoplasmic extracts were resolved by
SDS-PAGE and analyzed by Western blot.  Cytoplasmic
extract from IM9 cells was loaded on the same gel for
comparison (left lane).

FIGURE II-3.

25   INDUCTION OF CAT ACTIVITY BY hGR

Subconfluent CV-1 cells were cotransfected
with either pRSVgal (control), pRShGR alpha, or pRShGR
beta and the reporter plasmid pMTVCAT and cultured for
2 days in the presence (+) or absence (-) of 10 nM

30   dexamethasone.  CAT assays were performed as indicated
in Experimental Procedures (see Section II. F.).  C,
chloramphenicol; AC, 3-acetylchloramphenicol.

FIGURE II-4.

DOSE-RESPONSE TO DEX AND TITRATION OF pRShGR ALPHA

35   (A) CV-1 cells cotransfected with pRShGR alpha
and pMTVCAT (10 micrograms each plasmid) were cultured
in the presence of increasing concentrations of

encode proteins having at least about 10% of the transcription-activating properties characteristic of human glucocorticoid receptor; those DNA's include I9, I37, I102, I120, I204, I214, I262, I289, I305, I346, I384, I403, I408, I422, I428, I440, I448, I490, I515, I532, I550, and I684.

Construction of pRShGR-alpha is detailed in the part of the specification labeled "Experimental Section II". (See especially subsection II. F. (b), "Recombinant Plasmids".) Experimental Section II also details construction and properties of the pRShGR-alpha modifications referred to in the preceding paragraph.

With regard to the cDNA sequence for hGR shown in Figs. I-2(1) and I-2(2), the two C's at the 5'-end of the indicated sequence are part of the KpnI site joining the indicated segment to the 3' end of the segment which includes the RSV-LTR, and the T at the 3'-end of the indicated sequence is a few bases 5' of the point where the indicated segment is joined to the segment which includes the SV40 polyadenylation signal.

pRShMR was constructed in essentially the same manner as pRShGR-alpha and is essentially the same as pRShGR-alpha. Stated another way, with the exception of minor modifications at the insertion sites, the hMR segment shown in Fig. IV-2(A) replaces hGR, the sequence of which is shown in Figs. I-2(1) and I-2(2). Like pRShGR-alpha, pRShMV contains the receptor protein DNA coding sequence under the control of the promoter from Rous Sarcoma virus, plus the SV40 origin of replication. See Footnote 41 in the Reference portion of Experimental Section IV; also see Fig. IV-4(A); compare with Fig. II-1.

With regard to the hMR sequence shown in Fig. IV-2(B)-1 and IV-2(B)-2, the AG at the 5'-end of the segment is a few base pairs downstream of a HindIII site, whereby the hMR segment is joined to the

dexamethasone. The apparent $ED_{50}$ value for DEX was 3
nM. The levels of CAT activity were plotted as
percentages of the maximal response observed in a

5　particular experiment. No CAT activity was detected in
the absence of DEX.

(B) Titration. Increasing amounts of pRShGR
alpha were cotransfected into subconfluent CV-1 cells
with a constant amount of pMTVCAT (5 micrograms). The

10　plasmid pBR322 was used as carrier DNA to yield a total
of 30 micrograms DNA per plate. Cells were cultured
for 2 days in the presence of 10 nM DEX and CAT
activity was measured and plotted as in (A).
FIGURE II-5.

15　LOCATION OF FUNCTIONAL DOMAINS IN hGR

The hGR is schematically represented with
putative domains involved in transcription activation,
$tau_1$, and $tau_2$, indicated by hatched areas. The
DNA-binding domain is represented by a stippled box;

20　the steroid-binding domain, by a dotted box. The
positions of BamHI linker insertions are indicated by
triangles and circles. The numbers refer to the amino
acid position (see Experimental Section I) after which
the insertion occurs. Open symbols represent mutants

25　capable of inducing hormone-dependent transcriptional
activity at wild-type levels, as measured by CAT
activity, and closed symbols indicate greatly
diminished or abolished function. The bar indicates
the location of the divergent amino acids present in

30　hGR beta, which is not functional.
II. F. EXPERIMENTAL PROCEDURES
(a) CULTURE CONDITIONS

CV-1 and COS-1 cells were grown at 37°C in
Dulbecco's modified Eagle's medium supplemented with 5%

35　(v/v) fetal calf serum, 400 microg/ml ampicillin, and
100 microg/ml streptomycin. Cells were passed every 3
days and never allowed to reach confluency in order to

obtain good transfection efficiency.  All transfected
cultures were maintained at 37°C with 5% $CO_2$.

(b) RECOMBINANT PLASMIDS

5      Plasmid pRShGR alpha and pRShGR beta, which
direct the synthesis of the two forms of hGR in CV-1
and COS cells, were constructed from three DNA
fragments.  The first fragment is derived from pRSVCAT
(Gorman, et al., 1982b) and contains the RSV LTR,
10   pBR322 sequences, and SV40 polyadenylation site.  To
obtain this fragment, pRSVCAT was cut with HindIII and
the ends repaired by treatment with the Klenow fragment
of DNA polymerase I.  KpnI linkers were added to these
ends by standard procedures (Maniatis, et al., 1982),
15   and the plasmid was subsequently cut with HpaI, which
removed the CAT coding sequence.  The second fragment
contains the coding sequence of either hGR alpha or hGR
beta.  Plasmids pOB113 and pOB117 (S.M. Hollenberg,
unpublished results), which contain the entire coding
20   sequences of the alpha and beta form of hGR,
respectively, were cut with BamHI.  The ends were
repaired with Klenow, and the plasmids were cut with
KpnI.  Ligation of the first and second fragments
created plasmid pRhGR alpha and pRhGR beta.  The third
25   fragment to be added consists of a PvuII-HindIII
fragment containing the SV40 ORI obtained from the
plasmid pSV2CAT (Gorman, et al., 1982a).  The ends of
this fragment were repaired by treatment with Klenow,
and NdeI linkers were added to them.  This DNA
30   fragment, containing the SV40 ORI, was then introduced
into the single NdeI site present in pRhGR alpha and
pRhGR beta.  Finally, the single BamHI site present in
these plasmids was destroyed and replaced with a XhoI
site by insertion of a synthetic adaptor.  The
35   resulting plasmids are pRShGR alpha and pRShGR beta.
Plasmids pMTVCAT and pMTIaCAT were gifts from S. Gould.

(c) INSERTIONAL MUTAGENESIS

Insertion of amino acids disrupting the
wild-type sequence of hGR alpha was performed by the
5   following method.  Full-length linear pRShGR alpha DNA
was generated by partial digestion with restriction
enzymes AluI, DpnI, and BstNI.  In the case of DNA cut
by BstNI, the ends were first repaired with Klenow. The
DNA molecules were then fractionated by agarose gel
10   electrophoresis, and the linear form of the plasmid was
extracted.  BamHI linkers of 8- or 12-mer were added to
restore the original reading frame of the hGR amino
acid sequence.  Plasmids carrying a single BamHI linker
in the coding region of hGR were sequenced (Maxam and
15   Gilbert, 1980) to confirm the position of the linker
and the integrity of the hGR mutants.

(d) CELL TRANSFECTION AND CAT ASSAY

The recombinant DNA constructs were introduced
into CV-1 cells by calcium phosphate coprecipitation
20   (Wigler, et al., 1979) or into COS cells by
DEAE-dextran (Deans, et al., 1984).  Each plasmid
preparation used for transfection was purified using
two consecutive CsCl-EtBr equilibrium gradients.  After
transfection with the CAT gene constructs, CV-1 cells
25   were prepared for CAT assay as described by Gorman, et
al. (1982a).  The assays were performed with one third
of the total cellular extract and an incubation time of
6 hr.

(e) WESTERN BLOT ANALYSIS

30   Crude extracts from COS cells were prepared by
lysis with a buffer containing 10 mM Tris-CHl (pH 7.5),
100 mM NaCl, 1 mM EDTA, 0.5% Triton X-100.  Equal
amounts of protein (100 micrograms) were resolved by
7.5% polyacrylamide gel electrophoresis, transferred to
35   a nitrocellulose filter, and probed with anti-hGR
antibody GR884 (Harmon, et al., 1984), followed by
$^{125}$I-labeled Staphylococcus aureus protein A.  Filters

were air dried and exposed to film.  The amount of
receptor was quantitated by scanning the
autoradiographs.

5       (f) STEROID BINDING ASSAY

COS cells were lysed in hypotonic buffer
containing 10 mM Tris-HCl (pH 7.5), 10 mM NaCl, 1 mM
EDTA, 5 micrograms/ml antipain, 5 micrograms/ml
leupeptin, and 0.5 mM PMSF by Dounce homogenization and
10      centrifuged 10 min. at 15,000 x g to yield the
cytosolic fraction.  Incubations were performed in
hypotonic buffer adjusted to 100 mM NaCl and contained
100 micrograms of protein from the cytosolic fraction
and $2 \times 10^{-8}$ [$^3$H]DEX (Amersham, 95 Ci/mmol) in total
15      volume of 200 microliters.  Nonspecific binding was
measured by the addition of $2 \times 10^{-6}$ unlabeled DEX.
Reactions were carried out at 0°C for 2 hr., followed
by a 5 min. incubation with 20 microliters of 50%
dextran-coated charcoal (10:1 activated
20      charcoal:dextran) and centrifugation at 15,000 x g for
2 min. at 4°C.  Supernatants were counted by liquid
scintillation methods in a Beckman LS-7800 liquid
scintillation spectrophotometer.  Each assay usually
gave 2500-3000 cpm [$^3$H] labeled steroid; unlabeled DEX
25      competed for 70% of this binding.

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## EXPERIMENTAL SECTION III
## THE C-erb-A GENE ENCODES A
## THYROID HORMONE RECEPTOR
### III. A. SUMMARY AND INTRODUCTION

5

The human glucocorticoid receptor (hGR)
complementary DNA has been sequenced (see Weinberger,
et al., 1985b and Experimental Section I) and shown to
be functionally active (see Experimental Section II).

10  Interestingly, sequence analysis of the receptor showed
it to be related to the product of the v-erb-A oncogene
product of avian erythroblastosis virus (AEV) (see
Weinberger, et al., 1985). This led to the proposal
that the steroid receptors and the erb-A oncogene

15  products share a common primordial archetype and that
the erb-A proto-oncogene products may also be proteins
that bind to DNA enhancer elements. Recent
characterization of the human oestrogen (Green, et al.,
1986 and Greene, et al., 1986), chicken progesterone

20  (Jeltsch, et al., 1986 and Conneely, et al., 1986) and
human aldosterone (J. Arriza, C.W. and R.M.E.,
unpublished data) receptors further support these
conclusions.

Accordingly, we started characterizing the

25  human c-erb-A proto-oncogene even though its functional
identification could not be ensured. During the
progress of these studies, advances were made in
detailing the functional domains of the glucocorticoid
receptor (See Experimental Section II) which indicated

30  that the hGR hormone binding domain was unusually
large, encompassing the carboxy-terminal 300 amino
acids. This entire region has distant but significant
similarity to the carboxy terminus of v-erb-A which
therefore focused our attention on classes of molecules

35  that might exert transcriptional regulatory effects
similar to those of steroid hormones.

The molecular mechanism of thyroid hormone

stimulation of gene expression seems to be similar to
that outlined for steroids (see Eberhardt, et al.,
1980). Thyroid hormone is present in all chordate
5    species examined and exerts profound effects on
development and differentiation, such as metamorphosis
in amphibians (see Eberhardt, et al., 1980). Like
steroids, thyroid hormones may enter cells by passive
diffusion and bind to high affinity nuclear receptors
10   which in turn mediate a rapid and selective activation
of gene expression (Tata, et al., 1966 and Oppenheimer,
et al., 1972). Evidence favoring this hypothesis has
come largely from studies of the induction of growth
hormone and its messenger RNA in the somatotroph of the
15   rat anterior pituitary and in a number of related rat
somatotrophic cell lines (Samuels, et al., 1973 and
Tsai, et al., 1974). Thyroid hormones rapidly increase
the transcription of the growth hormone gene in these
cells (Martial, et al., 1977 and Evans, et al., 1982).
20   The increase in transcription is accompanied by
increased levels of nuclear thyroid hormone-receptor
complexes, is time- and concentration-dependent, and is
independent of protein synthesis (see Samuels, et al.,
1976; Spindler, et al., 1982; and Yaffe, et al., 1984).
25          The similarity of steroid and thyroid hormone
actions led us to examine the possibility that the
erb-A protein may itself be the thyroid hormone
receptor. We first isolated and characterized a cDNA
for human c-erb-A. The sequence predicts a 456 amino
30   acid polypeptide containing a cysteine/lysine/
arginine-rich region similar to the putative
DNA-binding domain of steroid hormone receptors and a
carboxy-terminal region distantly related to the
steroid binding domain. Using the functional assay
35   developed for analyzing the hormone binding properties
of cloned steroid receptors (see Experimental Section
I), we demonstrated that the translation product from

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the human c-erb-A cDNA possesses intrinsic thyroid
hormone binding activity, characteristic of the native
thyroid hormone receptor molecule.

### III. B. CHARACTERIZATION OF c-erb-A cDNA's

5          To isolate a human c-erb-A cDNA, a 500-base
pair (bp) PstI DNA fragment isolated from a region of
the AEV genome containing only the v-erb-A gene
(Vennstrom, et al., 1980) was used as a $^{32}$P-labeled
probe to screen two human placenta cDNA libraries.  Two
10   overlapping lambda gt10 cDNA clones were obtained by
screening two independent libraries of ~$10^6$ phage
recombinants each.  The restriction maps were deduced
for each of the cDNA clone EcoRI inserts from pUC8
subclones, pheA4 and pheA12 (Fig. III-1(A)).  Nucleo-
15   tide sequence analysis of these overlapping 1.5 kilo-
base (kb) cDNA clones revealed that the composite se-
quence contains a long open reading frame of 456 amino
acids with a presumptive initiator methionine codon at
nucleotide number 301 and a terminator codon at 1,669
20   in the sequence (Figs. III-1(B)-1 and III-1(B)-2).
Seven codons upstream of the ATG is an in-frame termin-
ator TAA providing support for the initiator methio-
nine, although another methionine found 26 codons down-
stream makes this assignment tentative.  No consensus
25   polyadenylation addition signal (AATAAA, see Proudfoot,
et al., 1976) is discernible in the 27 nuc- leotides
between the terminator and poly(A) tract in pheA12.

          A predicted polypeptide of relative molecular
mass 52,000 ($M_1$=52K), encoded within the human c-erb-A
30   cDNA translational open reading frame, shares 82% amino
acid identity with the region downstream from the viral
gag sequences (Debuire, et al., 1984) in AEV.  No gaps
in the amino acid comparison were revealed.  The human
c-erb-A amino acid sequence is homologous with the
35   viral protein beginning at viral amino acid residue 37
(Fig. III-2).  The carboxy-terminus of c-erb-A differs

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from that of v-erb-A in the following manner: amino
acid sequence similarity of the two polypeptides
terminates at residue 445 of c-erb-A and residue 380 of
5   v-erb-A (Fig. III-2).

        Alignment of the human c-erb-A nucleic acid
sequence with that of v-erb-A shows that ~74% of the
human gene is identical to the viral gene in the region
of amino acid homology (nucleotides 563-1636 in
10  c-erb-A, data not shown).  These comparisons, coupled
with previous data describing other human erb-A genes
mapping to chromosome 17 (Jansson, et al., 1983; Spurr,
et al., 1984; and Dayton, et al., 1984), indicate that
the human placenta c-erb-A gene we have isolated is
15  distinct.  One of the two chromosome 17 erb-A genes
(both of which we propose to call hc-erb-A alpha) has
82% similarity with the v-erb-A gene by nucleotide
sequence and 89% by amino acid identity (See Dayton,
1984).  Therefore, the placenta c-erb-A cDNA, which we
20  propose to call hc-erb-A beta, is more distantly
related to the viral erb-A gene than the hc-erb-A alpha
genes.

        Amino acid sequence comparisons between the
viral and cellular erb-A protein products and the
25  glucocorticoid receptor indicate graded levels of
homology with the carboxy-terminal half of the hGR
(Fig. III-2).  The highest degree of similarity is
found in a cysteine-rich sequence of 65 amino acids
beginning at c-erb-A amino acid residue 102 (see
30  Weinberger, et al., 1985).  There is 47% amino acid
identity in the comparison with the hGR and 52%
identity when c-erb-A is compared with the human
oestrogen receptor (hER) amino acid sequence (Fig.
III-2).  We have proposed that this region of the hGR
35  represents the DNA binding domain[4].  Mutagenesis and
expression studies have provided direct evidence for
its role in transcriptional activation (see

Experimental Section II).  Regions downstream from the
cysteine-rich domain, which correspond to the hormone
binding domain, contain reduced yet significan- (17%)
homology to the hGR and hER as found previously with
5    the viral erh-A product (see Weinberger, et al., 1985;
Green, et al., 1986; and Greene, et al., 1986).

### III. C. MULTIPLE erb-A GENES

Hybridization of restriction
endonuclease-digested human placenta DNA with a labeled
10   DNA fragment derived from the cysteine-rich region of
the c-erb-A polypeptide (Figs. III-1(A), III-1(B)-1 and
III-1(B)-2)) produced two bands in every digestion with
the exception of PvuII (Fig. III-3(A)).  The greater
intensity of the 9.4 kb-band suggested that it contains
15   two hybridizing DNA fragments.  When the hybridization
conditions were relaxed, additional bands were observed
in the products of each enzyme digestion.  For example,
two faint bands of 5.1 and 3.6 kb were seen after PstI
digestion (Fig. III-3(B)).  The hybridization probe
20   contains a single internal PstI site (Fig. III-1(A))
which probably explains the increased number of PstI
bands detected with this probe.

Similar high-stringency hybridization
experiments were performed using a 260 bp EcoRI-BamHI
25   fragment from the 5' untranslated region of pheA4 (Fig.
III-1(A) and data not shown).  Two hybridizing DNA
fragments were detected with all restriction enzymes
providing further support for the existence of two
related c-erb-A genes.  No additional DNA bands were
30   seen when the hybridization was performed under relaxed
conditions using this probe (data not shown).  We
conclude that there are two closely related hc-erb-A
beta proto-oncogenes and a third, less similar one, in
the human genome.

35   Laser-sorted chromosomes were prepared from
human lymphoid cells (Lebo, et al., 1984), bound to

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nitrocellulose filters and hybridized under
non-stringent hybridization and washing conditions
using the 1.5-kb EcoRI insert from pheA4 (Fig.
III-3(C)).  This probe detected only human chromosome
5    3-specific DNA and suggests that the three c-erb-A
beta-related genes are chromosomally linked, although
we cannot strictly exclude the possibility that the
non-stringently hybridizing erb-A gene maps in another
chromosome.  Interestingly, another steroid
10   receptor/erb-A genomic fragment has recently been
identified by characterizing the integration site for
hepatitis B virus in a human hepatocellular carcinoma
(Dejean, et al., 1986).  This locus has also been
mapped on human chromosome 3 suggesting that the erb-A
15   genes may be closely linked.

### III. D. EXPRESSION OF c-erb-A GENES

Northern blots hybridizing cytoplasmic
poly(A)-containing RNA's, isolated from various human
cell lines or human term placenta, with a 650-bp
20   BamHI-PstI fragment from the pheA4 (Fig. III-1(A))
revealed a single RNA species of 2,000 nucleotides that
is most abundant in HeLa and MCF-7 cells (Fig.
III-4(A)).  The size of the mRNA indicates that we have
isolated a nearly full-length c-erb-A cDNA.  HT1080
25   cells contain a small amount of the 2-kb transcript
while it is undetectable in IM-9 cells.  Human placenta
appears to contain multiple species of 5, 3 and 2.5 as
well as 2.0 kb RNA.  It is unclear whether the multiple
placenta bands represent nuclear precursors, or mature
30   mRNA's from a single gene, or the products of other
erb-A genes.

The protein products of the human c-erb-A cDNA
were characterized by in vitro translation.  A cDNA
containing the entire c-erb-A coding region was
35   inserted into the EcoRI site of the expression vector
pGEM3 in both orientations.  Capped RNA transcripts

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synthesized by T7 polymerase from these templates were
used to program protein synthesis in rabbit
reticulocyte lysates and the $^{35}$S-methionine-labeled
products were separated on SDS-polyacrylamide gels
5    (Laemmli, 1970).  Proteins with $M_r$ of 55, 52, and 35K
were detected when peA101 DNA (erb-A sense transcripts)
was used as template (Fig. III-4(B), lanes 3 and 4) but
not when peA102 DNA (erb-A antisense transcripts) was
used (Fig. III-4(B), lane 2).  The 55 and 52K products
10   may correspond to polypeptides initiating translation
at methionines 1 and 27, respectively (Fig.
III-1(B)-1), while the 35K product may be a proteolytic
breakdown product.

### III. E. THYROID HORMONE BINDING

15           Structural similarity of the steroid ligands
as well as the partial amino acid sequence homology
(40%) between the carboxy termini of the hGR and hER
(which specify the hormone binding domains; see Krust,
et al., 1986) support the hypothesis that the steroid
20   receptors comprise a family of regulatory proteins. The
more distant homology between the carboxy terminus of
c-erb-A and the steroid receptors suggested that erb-A
proto-oncogenes probably do not encode steroid
receptors, but is consistent with the hypothesis that
25   erb-A may respond to other molecules.  We have shown
that in vitro translation can be used as a means to
characterize hormone binding activity of cloned steroid
receptors (see Experimental Section I).  Accordingly,
this assay was employed to identify the putative
30   c-erb-A ligand.

             Steroid and thyroid hormones exert their
effects through fundamentally similar mechanisms.  To
address the possibility that erb-A may be the thyroid
hormone receptor, the in vitro translation products
35   were mixed with $^{125}$I-3,5,3'-triiodo-L-thyronine
($^{125}$I-T$_3$) in a hormone binding reaction (see Samuels,

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et al., 1974).  Nonspecific hormone binding was
determined by adding a 500-fold molar excess of cold $T_3$
to parallel samples.  Remarkably, the mixture
containing the 55 and 52K polypeptides (peA101)

5    acquired $125I-T_3$ binding whereas the anti-sense
RNA-programmed lysates (peA102) had only background
binding.   Hormone binding was sensitive to proteases
but not nucleases (data not shown).  Affinity of $T_3$ for
the cloned erb-A protein was determined by Scatachard

10   analysis (Fig. III-5(A)).  A $K_d$ value of $5 \times 10^{-11}$ M
was obtained, which is virtually identical to the $T_3$
binding ($6 \times 10^{-11}$ M) in HeLa cell nuclear extracts
(data not shown).

          Specific analogues of thyroid hormones have

15   characteristic competition patterns for $T_3$ binding to
the native thyroid hormone receptor.  We determined
whether the erb-A product synthesized in vitro shared
the same intrinsic hierarchy of affinities for a range
of natural and synthetic thyroid hormones.  Competition

20   of $125I-T_3$ binding was most effectively achieved with
3,5'; 3'-triiodothyroacetic acid (TRIAC) which
inhibited 50% of $125I-T_3$ binding at 300 pM (Fig.
III-5(B)).  In addition, L-thyroxine (Fig. III-5(B)),
D-$T_3$ and reverse $T_3$ (3,3',5'-triiodo-L-thyronine)

25   competed more poorly than $T_3$ (Fig. III-5(C)), while 100
microM vitamin $D_3$, aldosterone, cortisol, testosterone,
progesterone or oestradiol did not compete (data not
shown), consistent with the biochemical properties of
the rat thyroid hormone receptor (see Samuels, et al.,

30   1974; Samuels, et al., 1979; and Latham, et al., 1976).
          High salt (0.4 M KCl) HeLa cell nuclear
extracts contained thyroid hormone ($125I-T_3$) binding
activity, while none was found in cytoplasmic or
low-salt (0.1 M KCl) nuclear extracts (data not shown).

35   Competition of thyroid hormone binding in the nuclear
extracts was quantitatively similar to that of lysates

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containing c-erb-A made in vitro (compare Fig. III-5(B)
and (D)). Furthermore, thyroid hormone binding using
0.4 M KCl nuclear extracts from IM-9 cells, which
contain undetectable levels of c-erb-A mRNA (Fig.

5  III-4(A), lane 4), is negligible when compared with
similar HeLa extracts (data not shown). These results
provide direct evidence that c-erb-A is the thyroid
hormone receptor.

### III. F. CONCLUSIONS

10  The data in this section provide three
criteria that identify the c-erb-A product as the
thyroid hormone receptor. First, the overall
structural homology of c-erb-A is likely to be a
ligand-responsive regulatory protein. Second, the

15  expressed protein product has the same intrinsic
hierarchy of affinities for natural and synthetic
thyroid hormones as the native receptor. Third, the
molecular weights of erb-A in vitro translation
products are similar to the photo-affinity-labeled rat

20  thyroid hormone receptor (Pascual, et al., 1982). The
identity of erb-A as the thyroid hormone receptor could
be further substantiated by demonstrating its
transcriptional regulation of $T_3$-responsive genes such
as the growth hormone gene.

25  Analysis of the hGR and hM has revealed the
proteins to be composed of a series of functional
domains. (See Experimental Sections I and IV, and
Weinberger, et al., 1985a; also see Carlstedt-Duke, et
al., 1982; Dellweg, et al., 1982; Reichman, et al.,

30  1984; and Sherman, et al., 1978.) These include a
cysteine-rich region which contains structural
similarity to a repeated cysteine-rich region which
contains structural similarity to a repeated
cysteine-rich sequence found in Xenopus 5S gene

35  transcription factor IIIA and other transcriptional
regulatory proteins (Miller, et al., 1985; Berg, 1985)

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as well as carboxy-terminal sequences, which encode the
steroid-binding domain (see Experimental Section II and
Weinberger, et al., 1985a; also see Kumar, et al.,

5      1986).  Extension of this analogy to the thyroid
hormone receptor would predict its hormone binding
region to be localized near the carboxy-terminal end of
the molecule (Fig. III-6).  Putative DNA binding
sequences would be found in the cysteine-rich region

10     (Fig. III-6) where DNA binding properties of the hGR
and hER appear to be localized (see Experimental
Section II, and Kumar, et al., 1986; also S. Hollenberg
and R.M.E., unpublished data).

### III. G. THYROID HORMONE RECEPTOR AND ONCOGENESIS

15            Expression of the v-erb-A product in avian
erythroblasts is required for maintenance of the fully
transformed phenotype (see Graf, et al., 1983;
Frykberg, et al., 1983; Sealy, et al., 1983; and Kahn,
et al., 1986).  Chickens infected with viruses lacking

20     the v-erb-A gene display a less virulent disease, while
in vitro these infected erythroblasts differentiate
spontaneously and grow only with complex media
supplements.  Cells infected with erb-A+/erb-B+ virus,
however, have an increased capacity for self-renewal

25     and display a less differentiated phenotype. Structural
alterations of the v-erb-A protein could give rise to a
product exerting aberrant properties of growth control.
For instance, changes at the carboxyl terminus might
affect thyroid hormone binding activity, as has been

30     shown for the beta form of the human glucocorticoid
receptor, resulting in a constitutively active molecule
(see Experimental Sections II and III; also see
Weinberger, et al., 1985a), where changes abolish
steroid binding activity.  Insertional mutants in this

35     domain also inactivate steroid binding properties (see
Experimental Section II).  However, deletion of the
hormone binding region gives rise to a

constitutively active receptor indicating that this domain plays a modulatory role in transcriptional activation (V. Giguere and R.M.E., unpublished da·a).
These data lead us to predict that v-erb-A is unlikely
5   to bind hormone and is rather a constitutively active form of the thyroid hormone receptor.  The identification of erb-A as the thyroid hormone receptor provides the first direct evidence of a causative involvement of enhancers and their binding proteins in
10  oncogenic transformation.

III. H. A SUPERFAMILY OF REGULATORY GENES

Similarity of the steroid receptors with the v-erb-A oncogene product was sufficient to allow us to propose that both have evolved from a primordial
15  receptor gene (Weinberger, et al., 1985a).  Two surprising results have emerged from the studies presented here.  The first is that the occurrence of a family of erb-A proto-oncogenes implies the existence of one or more other molecules closely related to the
20  thyroid hormone receptor.  Physiological studies have not predicted the existence of a second class of thyroid hormone receptors and thus the characterization of this family may shed new light on mechanisms of developmental and homeostatic regulation.  The second
25  surprising observation from these results is the close kinship of the thyroid hormone receptors with the steroid hormone receptor family.  This relationship indicates that these molecules may all be part of a superfamily of regulatory proteins that have arisen
30  over evolutionary time to match the increasing developmental and physiological demands of more complex eukaryotes.

III. I. DETAILED DESCRIPTION OF FIGURES
REFERRED TO IN EXPERIMENTAL SECTION III
35  Figure III-1 (A) and (B).

Restriction map and sequencing strategy (A)

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and nucleotide and predicted amino acid sequence (B) of human placenta c-erb-A cDNA.  a, Orientations of the two subclones pheA4 and pheA12 relative to the composite restriction map.  Common restriction endonuclease cleavage sites are above the linear map. Thin lines, untranslated sequences; hatched box, erb-A coding region; arrows, DNA fragments sequenced.  b, Nucleotide sequence of the composite erb-A cDNA is presented in the 5' to 3' orientation.  The translational open reading frame related to the viral erb-A protein [21] is shown above the nucleotide sequence.  Adenosine residues (~130) are found at the 3' end of pheA12.  Numbers above the translated sequence indicate amino acid residues and nucleotide numbers are on the right of the sequence.

Figure III-1 (A) and (B) Methods

Recombinant phage (~$10^6$) from each of two human placenta lambda gt10 cDNA libraries (Huynh, et al., 1985) were screened using a nick-translated (Rigby, et al., 1977) 500-bp PstI fragment isolated from pAEV-11 (Vennstrom, et al., 1980)).  The hybridization mixture contained 50% formamide, 1 x Denhardt's, 5 x SSPE, 0.1% sodium dodecyl sulphate (SDS), 100 microgram $ml^{-1}$ denatured salmon sperm DNA and $10^6$ c.p.m. $ml^{-1}$ of $^{32}P$-labeled PstI fragment (specific activity = 1 x $10^8$ c.p.m. $microgram^{-1}$). Duplicate nitrocellulose filters were hybridized at 37°C for 18 h, washed three times for 20 min each in 0.1 x SSC, 0.1% SDS (1 x SSC = 150 mM NaCl, 15 mM trisodium citrate) and autoradiographed at -70°C with an intensifying screen.  Two hybridization-positive clones were isolated, subcloned into the EcoRI site of pUC8, and sequenced by the chemical cleavage method (Maxam, et al., 1977).

Figure III-2.

Amino acid sequence comparison between the

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carboxy-terminal portions of the v-erb-A oncogene
product, the human placenta c-erb-A polypeptide and the
human glucocorticoid and oestrogen receptors
Translated amino acid sequences for both the v-erb-A
5   protein (upper sequence) and the human placenta c-erb-A
polypeptide (second sequence) were compared by aligning
matching residues.  A computer program for the
concurrent comparison of three or more amino acid
sequences (Johnson, et al., 1986) was used to align
10  c-erb-A human glucocorticoid receptor (hGR third
sequence from top; see Experimental Section I, Figures
I-2(1) and I-2(2)) and human oestrogen receptor (hER,
bottom sequence; see Green, et al., 1986)
carboxy-terminal amino acid sequences, on the basis of
15  progressive evaluation of selected segments from each
sequence. Amino acid residues matched in at least three
of the polypeptides are boxed.  Amino acid matches
between the two erb-A polypeptides are indicated by an
asterisk above the top sequence in each column.  Amino
20  acid identities between the steroid receptors are
designated by crosses below the sequences.  Hyphens,
gaps inserted to maximize the number of matches in the
comparison. Cysteine residues conserved between the
four polypeptides printed white-on-black.
25  Figure III-3 (A), (B) and (C).
             Southern analysis and chromosome mapping of
human placenta DNA with c-erb-A DNA probes.  A, Human
term placenta DNA was digested with restriction
endonucleases and products were separated on a 0.8%
30  agarose gel.  DNA's were transferred to nitrocellulose
paper (Southern, 1975) and hybridized in 50% formamide,
5 x SSPE, 1 x Denhardt's, 0.1% SDS, 100 microgram ml$^{-1}$
salmon sperm DNA with the 450-bp SstI fragment from the
pheA4 which was nick-translated to a specific activity
35  of 5 x 10$^8$ c.p.m. microgram$^{-1}$.  The filter was washed
in 0.1 x SSC, 0.1% SDS at 60°C and exposed to X-ray

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film at -70°C with an intensifying screen.  Lambda
HindIII DNA markers (size in kb) are aligned to the
left of the autoradiogram.  B, Analysis of placenta DNA
using the same c-erb-A probe as in section a above,
5    under non-stringent hybridization conditions.  A
parallel blot containing the same samples was
hybridized as in section a, except that 35% formamide
was used.  The hybridized filter was washed in 2 x SSC,
0.1% SDS at 55°C and exposed to X-ray film as described
10   above.  C, Chromosome mapping of human c-erb-A genes.
Human lymphocyte chromosomes were separated by laser
cytofluorometry (Lebo, et al., 1984) and probed using
the non-stringent hybridization conditions described in
section b, but with the 1.5 kbp EcoRI insert from pheA4
15   as probe.

Figure III-4 (A) and (B).

        Human c-erb-A expression.  A, Northern
analysis of RNA's from human cell lines and human
placenta.  Cytoplasmic poly(A)-containing RNA (12
20   microgram) from HeLa, MCF-7 and IM-9 cells, or total
poly(A) RNA's from HT1080 or placenta, were separated
on a 1% agarose gel containing formaldehyde,
transferred to nitrocellulose (Thomas, 1980) and probed
using a nick-translated 650-bp BamHI-PstI pheA4
25   fragment.  Cytoplasmic RNA's were isolated from the
cell lines using isotonic buffer and 0.5% NP40, while
the placenta RNA's were extracted from fresh tissue
using guanidine thiocyanate (Chirgwin, et al., 1979).
Lane 1, HeLa; lane 2, HT1080, lane 3, human placenta;
30   lane 4, IM-9; lane 5, MCF-7.  B, Synthesis of erb-A
polypeptides in vitro.  The $^{35}$S-methionine-labeled
products synthesized using T7 polymerase-catalysed RNA
transcripts were separated on a 12.5%
SDS-polyacrylamide gel which was fluorographed
35   (EN$^3$HANCE, New England Nuclear).  Lane 1, control
(without mRNA); lane 2, peA102 (anti-sense RNA, 4

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microliters); lane 3, peAl01 (sense RNA, 1 microliter);
lane 4, peAl01 (4 microliters RNA).  Sizes of protein
standards: bovine serum albumin, 66.2K; ovalbumin, 45K
and carbonic anhydrase, 31K.

5    Figure III-4 (A) and (B) Methods.

The EcoRI insert from phoAl2 which contains
the entire coding region of c-erb-A was inserted into
the EcoRI site of pGEM3 (Promega Biotec) in both
orientations.  Plasmid DNA's peAl01 and peAl02 were
10   linearized with HindIII, purified on 0.8% agarose gels
and used as templates for T7 polymerase-catalysed
synthesis of RNA's in vitro (see Experimental Section
I).  After P60 chromatography, total nucleic acid
material (2 micrograms) was used to program protein
15   translation in a rabbit reticulocyte lysate system
(Promega Biotec) with $^{35}$S-methionine (25 microCi, 1100
Ci mmol$^{-1}$, New England Nuclear) final volume 25
microliters.

Figure III-5 (A), (B), (C) and (D).

20   Thyroid hormone binding to erb-A polypeptides
synthesized in vitro.  A, Scatchard analysis of $^{125}$I-T$_3$
binding to the erb-A polypeptides made in vitro.  The
erb-A polypeptides (2 microliters from the in vitro
translation mixture in a total volume of 2 ml) were
25   assayed for specific thyroid hormone binding activity
using hydroxylapatite to measure the amount of bound
and free labeled hormone at different concentrations of
$^{125}$I-T$_3$ as described (Gruol, 1980).  B, Competition of
thyroid hormone analogues for $^{125}$I-T$_3$ binding to erb-A
30   polypeptides synthesized in vitro.  Samples (2
microliters) from the peAl01 (sense strand)-programmed
reactions were used in $^{125}$I-T$_3$ standard binding
reactions (Samuels, et al., 1974) with increasing
concentrations of unlabeled thyroid hormone or
35   analogues to compete with labeled hormone. Specifically
bound thyroid hormone is plotted against

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concentration of competitor compound.  C, Competition
of triiodothyronine isomers from $^{125}I$-$T_3$ binding to
erb-A polypeptides synthesized in vitro.  Binding
reactions were performed as above adding increasing
concentrations of $T_3$ isomers.  Thyroid hormone bound to
erb-A is plotted on the ordinate.  D, Competition of
thyroid hormone analogues for $^{125}I$-$T_3$ binding to 0.4 M
KCl HeLa cell nuclear extracts.  HeLa cell nuclei were
extracted with a buffer containing 0.4 M KCl (Samuels,
et al., 1974).  The protein extract (25 micrograms)
(determined by Bio-Rad protein assay) was mixed with
0.6 nM $^{125}I$-$T_3$ in standard binding reactions with
increasing concentrations of thyroid hormone and
analogues (Samuels, et al., 1974; Latham, et al.,
1976).

Figure III-5 (A), (B), (C) and (D) Methods.

        Labeled $^{125}I$-3,3',5-triiodo-L-thyronine (New
England Nuclear, 2,200 Ci mmol$^{-1}$, 0.3 nM final) was
mixed with erb-A polypeptides synthesized in the in
vitro translation mixture (described in Fig. III-4(A))
in $T_3$-binding buffer (0.25 M sucrose, 0.25 M KCl, 20 mM
Tris-HCl (pH 7.5), 1 mM $MgCl_2$, 2 mM EDTA, 5 mM
dithiothreitol (DTT)) (Samuels, et al., 1974) at 0°C
for 2 h in a final volume of 250 microliters.  Specific
hormone binding was determined by adding a 1,000-fold
excess of unlabeled hormone and assayed by counting
radioactivity eluting in the excluded volume from a
Sephadex G-25 fine (Pharmacia) 0.9 x 4.0 cm column
(Samuels, et al., 1974).

Figure III-6.

        Schematic comparison of the steroid and
thyroid hormone receptors.  Amino-acid sequences of the
receptor molecules aligned in Fig. III-2 are
represented schematically.  CYS, cysteine-rich region
encoding the putative DNA binding domain found in the
receptor proteins (Cys-rich region residues are:

c-erb-A, 102-169; hGR, 421-486; hER, 185-250);
Cortisol, oestradiol and $T_3/T_4$, hormone binding regions
in the carboxyl termini; 1 MM, immunogenic region of
the human glucocorticoid receptor.  Numbers separating
boxes, percentage amino acid identities between the
receptor species in the intervals between the vertical
broken lines; hGR, human glucocorticoid receptor; hER
human oestrogen receptor; hc-erb-A beta, human thyroid
hormone receptor.

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## EXPERIMENTAL SECTION IV

### CLONING OF HUMAN MINERALOCORTICOID RECEPTOR COMPLEMENTARY DNA: STRUCTURAL AND FUNCTIONAL KINSHIP WITH THE GLUCOCORTICOID RECEPTOR

#### IV. A. SUMMARY

Low-stringency hybridization with human glucocorticoid receptor (hGR) complementary DNA was used to isolate a new gene encoding a predicted 107-kilodalton polypeptide. Expression studies demonstrate its ability to bind aldosterone with high affinity and to activate gene transcription in response to aldosterone, thus establishing its identity as human mineralocorticoid receptor (hMR). This molecule also shows high affinity for glucocorticoids and stimulates a glucocorticoid-responsive promoter. Together the hMr and hGR provide unexpected functional diversity in which hormone-binding properties, target gene interactions, and patterns of tissue-specific expression may be used in a combinatorial fashion to achieve complex physiologic control.

#### IV. B. INTRODUCTION

The hypothalamic-pituitary-adrenal axis integrates a variety of neuroendocrine inputs to regulate the synthesis and secretion of the adrenal corticosteroids. These steroid hormones exert effects on growth, development, and homeostasis by their interaction with intracellular receptor proteins that directly regulate the transcription of sets of target genes (1,2). Two receptor systems have been defined for the corticosteroids; these are termed the glucocorticoid receptor (GR) and the mineralocorticoid receptor (MR). Early functional assays classified the corticosteroids as either glucocorticoid, by their effect in promoting glycogen deposition in the liver, or mineralocorticoid, by their effect in promoting sodium retention by the kidney. However, each steroid

class is not restricted to interacting with only its
cognate receptor, and glucocorticoids, in particular,
can have substantial mineralocorticoid activity (1-3).

5      It is now evident that the MR has significant
in vitro affinity for both glucocorticoids and
mineralocorticoids (3,4). Since the circulating levels
of glucocorticoids are several orders of magnitude
higher than those of aldosterone, the primary

10 mineralocorticoid, glucocorticoid activation of the MR
may be functionally significant. Whereas the secretory
epithelia of tissues such as kidney and intestine
regulate electrolyte and water balance in response to
aldosterone, it is possible that additional mechanisms

15 confer these tissues with sensitivity to
mineralocorticoids (5). No clear functional role has
emerged for the MR expressed in other tissues, but
physiological responses in brain may result from
glucocorticoid interactions with the MR (5-7).

20      Despite the availability of high-affinity
radioactively labeled ligands, the MR has been
refractory to purification, and its biochemical
properties, in comparison to GR, remain poorly
understood. Application of the techniques of molecular

25 biology to the study of the MR would facilitate its
biochemical characterization and, eventually, an
understanding of the genes under its transcriptional
control and the roles their products play in
homeostasis.

30      Molecular cloning of the glucocorticoid (see
Experimental Section I and refs. 8,9), estrogen (10),
and progesterone (11) receptors has permitted the
determination of their primary amino acid structures
and prediction of functional domains common to this

35 family of regulatory proteins. Experimental dissection
of glucocorticoid (see Experimental Section II and ref.
12) and estrogen (13) receptors has revealed a

centrally located DNA-binding domain rich in cysteine,
lysine, and arginine, and a carboxyl-terminal region
where steroid hormones interact.  Functional studies of
5  GR suggest that hormone binding to the carboxyl
terminus unmasks the DNA binding region to permit
interactions of receptor with DNA and activation of
transcription (14, 15).  Comparison of the
cysteine-rich DNA-binding regions of steroid and
10  thyroid hormone receptors shows a high degree of
relatedness between these molecules (16).  The
invariant cysteine residues have led to the hypothesis
that coordination of $Zn^{2+}$ metal atoms maintains a
structural configuration for DNA binding analogous to
15  that proposed for _Xenopus_ 5S gene transcription factor
IIIA (17).  The steroid-binding regions of the steroid
receptor family also show substantial conservation
consistent with evolution of various receptor classes
from a common ancestral precursor (11, 16).
20          We have used the structural similarity between
steroid hormone receptors to isolate a gene product
closely related to the human glucocorticoid receptor
(hGR).  Nonstringent hybridization with an hGR probe
was used to isolate a human genomic DNA fragment highly
25  related to the hGR cysteine-rich sequence.  Using this
DNA as a probe, we obtained complementary DNA's
(cDNA's) that code for a molecule having a strong
homology with the hGR from the cysteine-rich region to
the carboxyl terminus.  When expressed in cells, this
30  molecule binds aldosterone with high affinity and
activates aldosterone-response transcription of the
long terminal repeat (LTR) of the mouse mammary tumor
virus (MMTV).  The overlap of the ligand and DNA
sequence specificities of this human mineralocorticoid
35  receptor with those of hGR suggest that the distinct
roles traditionally assigned to these regulatory
molecules should be reconsidered.

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## IV. C. ISOLATION OF hMR cDNA

For the identification of glucocorticoid
receptor-related genes, human placenta DNA was digested
with restriction endonucleases, fractionated by agarose
5   gel electrophoresis, and the fractions were hybridized
with hGR 1.2, an 1100-bp fragment of hGR cDNA
containing sequences encoding the DNA-binding domain
(see Experimental Section I; also ref. 15).  Southern
blot analysis revealed several distinct bands specific
10   to low-stringency hybridization conditions (compare
Fig. IV-1, (A) and (B)).  The 2.5-kilobase pair (kbp)
HindIII fragment (bracketed by asterisks in Fig.
IV-1(B)) was well resolved from other hybridizing bands
and was judged suitable for direct genomic cloning.
15   HindIII-digested DNA from human placenta was
preparatively size-fractionated on an agarose gel, and
the 2.5-kbp region was isolated for the construction of
a genomic library.  This lambda gt10 library was then
screened under conditions of low-stringency
20   hybridization with hGR 1.2 as the probe.  The insert
from one positive genomic clone, lambda HGH, was
nick-translated and used as a probe on a Southern blot
under high-stringency hybridization conditions (Fig.
IV-1(C)).  The 2.5-kbp HindIII signal corresponded to
25   that seen under nonstringent conditions, indicating
that a portion of the desired genomic fragment had been
isolated.  Sequence analysis of the insert from lambda
hGH revealed an exon of 140 base pair (bp) flanked by
intron sequences (Fig. IV-1(D)).  Overall this exon has
30   68 percent nucleotide identity with the homologous hGR
cDNA sequence, but a region conserving 85 nucleotides
out of 104 probably confers its cross-hybridization
properties.  This highly conserved region corresponds
to a portion of the hGR DNA-binding domain (15).  The
35   lambda HGH exon codes for 46 amino acids beginning with
16 nonconverted residues and followed by the first of

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the highly conserved cysteine residues characteristic
of steroid hormone receptors (8-11). Of the next 30
residues, 28 are identical to hGR. These analyses

5    demonstrated the isolation of a genomic fragment
containing a sequence related to, but clearly distinct
from, that found in the hGR cDNA sequence (see
Experimental Section I).

The insert from lambda HGH was used as a probe
to screen cDNA libraries for clones corresponding to

10   this hGR-related gene. Mineralocorticoid receptor was
considered a candidate to be encoded by such a gene.
Since kidney is known to be a mineralocorticoid-
responsive tissue, several human kidney cDNA libraries
were screened. Eleven positive clones were isolated

15   from these lambda gt10 libraries at a frequency of
three to four per $10^6$ recombinant phage. Two over-
lapping clones, lambda hk2 and lambda hk10, were sub-
jected to nucleotide sequence analysis and together
found to span 5823 nucleotides (Figs. IV-2(A),

20   IV-2(B)-1 and IV-2(B)-2). The exon-intron boundaries
of lambda HGH were verified by sequencing these cDNA
clones. The lambda hk10, encompassing nucleotides 1 to
3750, con- tains a large open reading frame predicting
the entire primary amino acid sequence. The DNA insert

25   from lamb- da hk2 extends from nucleotides 802 to 5823,
but con- tains an internal 351-bp deletion from 2235 to
2586. Three additional clones were examined and
determined to have the same structure as lambda hk10 in
the deleted region. It is likely that the deletion in

30   lambda hk2 represents either a cloning artifact or a
rare messenger RNA (mRNA) splicing error (18). The
sequence of the reported 3'-untranslated region down-
stream of nucleotide 3750 is derived from lambda hk2.
The composite sequence of these two cDNA's is termed

35   hMR (Fig. IV-2(A)). With the first in-frame ATG (posi-
tion 223) downstream of an in-frame termination codon

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(position 136), hMR has a 5'-untranslated region of at
least 216 nucleotides.  The sequence surrounding this
first ATG agrees with the consensus described by Kozak

5  (19).  This predicted initiator methionine codon begins
an open reading frame encoding 984 amino acids.
Following a termination codon (position 3175) is a
2.6-kb 3'-untranslated region with a typical
polyadenylation signal (AATAAA) found 17 nucleotides

10  upstream of a 70-nucleotide poly(A) (polyadenylated)
tract.  Long 3'-untranslated regions are a
characteristic feature of steroid hormone receptor
mRNA's (see Experimental Section I; also refs. 9-11).

### IV. D. THE DNA- AND HORMONE-BINDING REGIONS

15          The protein encoded by hMR cDNA has the
structural properties of a steroid hormone receptor
closely related to hGR.  Comparison of the predicted
amino acid sequence of hMR with that of hGR
demonstrated high degrees of homology with both the hGR

20  DNA binding and steroid binding domains.  The hMR gene
encodes a protein of 984 amino acids with a predicted
molecular size of 107 kD, significantly larger than the
777 residues of hGR.  This size discrepancy is
primarily due to the large amino terminus, which bears

25  no homology to hGR.  Considerable heterogeneity of size
and sequence for this region exists between the
receptors for glucocorticoid, estrogen, and
progesterone (see Experimental Section I; also refs.
9-11).  Amino acid homology begins in the centrally

30  located DNA region with 94 percent amino acid identity
in 68 residues (Fig. IV-3).  Separating the DNA-binding
domain and the carboxyl-terminal steroid-binding domain
is a region with relatively low sequence conservation
found between other steroid hormone receptors.  It has

35  been speculated that the region may serve as a
molecular hinge between the two domains (see
Experimental Section II and ref. 13).  Comparison with

hGR shows this region of hMR to contain an additional
24 amino acids including a sequence of 4 glutamines
followed by 8 prolines encoded by repetitive nucleotide
elements.  The significance of this unusual sequence in
terms of origin and function is unclear, but

5      structure-breaking prolines are consistent with a hinge
region.  A comparison of the carboxyl-terminal 250
amino acids of hMR with hGR shows 57 percent amino acid
identity as well as a number of conservative amino acid

10     substitutions.  Some of these substitutions may
preserve hydrophobic regions necessary for steroid
hormone interaction.

### IV. E.  EXPRESSION AND HORMONE BINDING

We have used transfection of the monkey kidney
15     cell line CV1 and its derivative (that is, SV40 T
antigen-transformed) cell line COS-1 (referred to as
COS) to study glucorticoid receptor function.  (See
Experimental Section II.)  High levels of polypeptide
expression from transfected hMR were essential to

20     facilitate steroid-binding experiments in transfected
cells.  Since plasmids containing the SV40 origin of
replication can replicate to high copy numbers in COS
cells, an expression vector for hMR coding sequences
similar to pRShGR alpha, used previously in hGR

25     studies, was constructed.  The plasmid, pRShMR,
contains the hMR coding sequence, under the control of
the promoter from Rous sarcoma virus, and the SV40
origin of replication (Fig. IV-4(A)).

Ligand specificity of the hMR protein was
30     determined by preparing cytosol extracts from COS cells
transfected with pRShMR.  Two days after transfection,
cells were harvested, and hormone binding was measured
by a dextran-treated charcoal assay.  Mock-transfected
control extracts had no specific binding activity for

35     [$^3$H]aldosterone, whereas extracts from
pRShMR-transfected cells found significant amounts of

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[3H]aldosterone with high affinity.  A dissociation
constant ($K_D$) of 1.3 nM for the binding of
[3H]aldosterone was determined by a Scatchard analysis
(Fig. IV-4(B)).  This value is in good agreement with

5      those reported for aldosterone binding to
mineralocorticoid receptor (2, 20).  Competition
experiments were then performed to examine the ability
of different unlabeled steroids to compete with 5 nM
[3H]aldosterone for binding when present at 1-, 10-, or

10     100-fold molar excess (Fig. IV-4, (C) and (D)).  This
provided a measure of the relative affinity of each of
these steroids for hMR.  The results of these
experiments show that aldosterone, corticosterone,
deoxycorticosterone, hydrocortisone (cortisol) all have

15     very similar affinities for hMR.  Dexamethasone,
progesterone, and spironolactone demonstrated weaker
binding affinity while estradiol competed very poorly
for binding to hMR.  Overall, this hierarchy of
affinities indicated that hMR encoded the human

20     mineralocorticoid receptor (2, 20).

### IV. F. TRANSCRIPTIONAL ACTIVATION

Steroid hormone action is characterized by
hormone-dependent modulation of target gene transcrip-
tion.  The assay for transcriptional regulation by

25     transfected hGR in CVI cells (see Experimental Section
II) was adapted to hMR (Fig. IV-5(A), (B) and (C)).
The expression plasmid used for steroid-binding assays,
pRShMR, was cotransfected with a reporter plasmid
called GMCAT, which contains the MMTV LTR linked to the

30     bacterial gene for chloramphenicol acetyltransferase
(CAT).  Thus CAT activity provides an enzymatic assay
for the transcriptional activity of the MMTV promoter.
The MMTV promoter contains several glucocorticoid
response elements (GRE's), enhancer-like DNA sequences

35     that confer glucocorticoid responsiveness via
interaction with the GR (21).  It was possible that

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hMR, because of the near identity of its DNA-binding
domain to that of hGR, might also recognize the MMTV
LTR.  When CV1 cells were cotransfected with pRShMR and
GMCAT, we observed full CAT activity.  This activity
5    was independent of added aldosterone suggesting that,
in contrast to transfected hGR, sufficient hormone was
present in serum (fetal calf serum, 5 percent) to fully
activate hMR (Fig. IV-5(B)).  In the presence of
charcoal-treated serum (22) CAT activity became
10   responsive to the addition of exogenous aldosterone
(Fig. IV-5(C)), indicating that hMR cDNA encodes a
functional steroid hormone receptor.  While the hMR was
also activated by the glucocorticoid agonist
dexamethasone, the hGR did not respond to even
15   supraphysiological concentrations (10 nM) of
aldosterone.

### IV. G. TISSUE-SPECIFIC EXPRESSION

We examined the expression of MR mRNA
homologous to hMR cDNA in rat tissues by Northern blot
20   hybridization (23).  Classical mineralocorticoid target
tissues such as kidney (24) and gut (25), as well as
tissues such as brain, pituitary, and heart, contained
mRNA homologous to hMR (Fig. IV-6).
Aldosterone-sensitive cells in kidney are primarily
25   restricted to the distal and cortical collecting
tubules (2), and therefore a modest level of expression
in this tissue was not unexpected.  High levels of MR
(type I corticosteroid-binding sites) have been
reported in rat brain, particularly in the hippocampal
30   formation (4, 6).  In comparing dissected hippocampal
RNA with RNA prepared from total brain, we found a
striking enrichment of message in the hippocampus.
While aldosterone binding has been reported for
pituitary (26), cultured aortic cells (27), and spleen
35   (28), no such activity has been reported in muscle.
Liver expresses GR, but has no detectable high-affinity

aldosterone-binding activity (29), and as would be
expected no hybridization to liver RNA was observed.
Reprobing of the same Northern blot with an analogous
portion of hGR cDNA demonstrated hybridization to mRNA
5   species of different sizes, and indicated that the MR
and GR do show differential patterns of tissue-specific
expression.

                    IV. H. CHROMOSOME MAPPING
10          To determine the chromosomal location of the
mineralocorticoid receptor gene, we tested hMR against
a panel of rodent-human somatic cell hybrids retaining
different combinations of human chromosomes (30).  The
DNA fragments specific for the mineralocorticoid
15  receptor gene segregated concordantly with human
chromosome 4 in 15 hybrid cell lines.  Discordant
segregation was observed for all other human
chromosomes, including chromosome 5, site of the
glucocorticoid receptor gene (see Experimental Section
20  I and ref. 31).  To confirm the assignment to
chromosome 4, we tested a restricted set of microcell
hybrids, each of which carry one to three human
chromosomes (32), for the hMR gene by Southern analysis
(Fig. IV-7).  Six EcoRI fragments detected by the
25  coding portion of lambda hk2 co-segregate with
chromosome 4 in this hybrid panel.  In particular, the
hMR gene is present in HDM-1132B, a cell line that
carries chromosome 4 as its only human chromosome.

                    IV. I. IMPLICATIONS FOR ADRENAL
                    CORTICOSTEROID PHYSIOLOGY
30          Human mineralocorticoid receptor cDNA encodes
a polypeptide that is highly homologous to the human
glucocorticoid receptor.  In the DNA-binding domain,
hMR maintains approximately 94 percent amino acid
35  identity to hGR while the steroid-binding domain
localized in the carboxyl terminus has 57 percent
identity.  The recently reported sequence (11) of the

rabbit progesterone receptor (rPR) also has a high
degree of relatedness to hMR.  Comparison of the amino
acid identity in hGR and rPR structural domains with

5   that of hMR (Fig. IV-8) demonstrates the remarkable
similarity of these functionally distinct regulatory
proteins.  The homology of hMR with rPR is almost
identical to the hGR-hMR comparison, with 90 percent of
the amino acids shared in the DNA-binding domain and 56

10  percent in the steroid-binding region.  In contrast, a
comparison of the same regions of hMR with human
estrogen receptor (10) indicates 56 percent identity in
the DNA binding domain and 21 percent sequence identity
in the steroid-binding carboxyl terminus.  The degree

15  of structural homology shared by hMR, hGR, and rPR, and
the structural relatedness of their ligands, suggests
that they may comprise a subfamily of steroid hormone
receptors.

          Expression of the hMR polypeptide in COS cells

20  by transient transfection permitted the evaluation of
its steroid-binding potential.  The results of these
analyses indicated that hMR cDNA encodes a human
mineralocorticoid receptor.  Scatchard analysis
demonstrated that extracts from cells transfected with

25  pRShMR bound [$^3$H]aldosterone with a $K_D$ of 1.3 nM, while
reported $K_D$ values for aldosterone binding to MR range
from 0.5 to 3 nM (2).  This is the single most
important criterion in defining this gene product as
the human mineralocorticoid receptor.  Steroid-binding

30  competition studies have further supported this
identification of hMR.  The mineralocorticoid
deoxycorticosterone and the glucocorticoids
corticosterone and cortisol compete as effectively as
aldosterone itself, whereas the synthetic

35  glucocorticoid dexamethasone and progesterone have
lower affinities for the hMR.

          The extensive amino acid sequence identity in

the presumptive steroid-binding domains of hMR, hGR,
and rPR is compatible with the similar ligand-binding
properties of these receptors.  The mineralocorticoid,
5   glucocorticoid, and progesterone receptors exhibit a
limited ability to discriminate between the similar
21-carbon atom structures of the mineralocorticoids,
glucocorticoids, and progestins.  This lack of
specificity is particularly relevant to the MR and GR.
10  For example, the MR binds glucocorticoids with an
affinity equal to that for aldosterone.  Indeed, it may
be that only in tissues such as kidney, where
additional mechanisms confer selective response to
aldosterone, does the MR function as a classical
15  mineralocorticoid receptor (3, 5).  The MR also binds
progesterone with a high affinity, but one lower than
its affinity for corticosteroids.  There is some
indication that progesterone may act as a partial
agonist or antagonist of mineralocorticoid action (33),
20  and it is not clear whether glucocorticoids act as full
agonists in binding to the mineralocorticoid receptor.
Similarly, the GR binds glucocorticoids with a $K_D$
between 20 to 40 nM and it binds aldosterone with a $K_D$
between 25 to 65 nM (2).  Therefore, the important
25  distinction between the hormone-binding properties of
MR and GR may not be one of ligand specificity, but
rather of a high-affinity versus a lower affinity
receptor for the corticosteroids.

The function of the hMR in vivo is complicated
30  by the serum cortisol-binding protein, transcortin.
This protein sequesters cortisol and, because of its
differential distribution, transcortin could influence
local glucocorticoid concentration.  High levels of
transcortin in kidney would reduce available cortisol
35  from plasma to favor aldosterone sensitivity, whereas
low levels of transcortin in the brain would suggest
that, in the central nervous system, glucocorticoids

may be the predominant hMR ligand. Thus, the preferred
physiologic ligand for hMR apparently varies depending
on the site of receptor expression (3). This model and
5   others (5) have been proposed to explain the
responsiveness of some tissues to aldosterone despite
much higher levels of competing glucocorticoids.

       The degree of homology between hMR and hGR in
the DNA-binding domain (only four amino acid residues
10   differ in this conserved 68-residue region) suggests
that these receptors may recognize similar regulatory
elements. The activation of the MMTV LTR by the
transfected hMR in response to both aldosterone and
dexamethasone supports this conclusion, although the
15   progesterone receptor has also been demonstrated to
regulate this promoter (21). Furthermore, differences
between hMR and hGR in the DNA-binding domain, or in
other regions such as the highly divergent amino
termini of these molecules, may influence target gene
20   specificity in ways not revealed in this assay.
However, we have utilized transcriptional regulation of
the MMTV LTR by hMR and hGR to examine their activation
by mineralocorticoids and glucocorticoids. While the
hMR response was approximately equivalent with either
25   10 nM aldosterone or dexamethasone, hGR was activated
by dexamethasone but was insensitive to aldosterone in
this assay. Transcriptional activation by hMR in
response to exogenous cortisol was also observed. These
data indicate that in transfected cells both
30   mineralocorticoids and glucocorticoids can activate
hMR-mediated gene transcription. On the basis of this
functional property, we conclude that the hMR is highly
responsive to adrenal corticosteroids and therefore may
function as a glucocorticoid receptor.

35        In addition to elucidating the pharmacologic
and physiologic function of the mineralocorticoid
receptor in coordinating response to corticosteroids,

the isolation of hMR cDNA will facilitate investigation
of the role of hMR in a number of disease states, among
them hypertension and pseudohypoaldosteronism (PHA). An
association of mineralocorticoids with hypertension has
5  been recognized for several decades, and it may be that
hMR-mediated sodium retention and increased blood
volume are, in part, responsible for some forms of
hypertension (34). PHA is an autosomal recessive
disorder characterized by lack of responsiveness to
10  normal or elevated aldosterone levels. Recent work has
demonstrated diminished or complete loss of
high-affinity aldosterone-binding sites in patients
with this disease (35) which is likely to result from a
mineralocorticoid receptor genetic defect. The
15  chromosomal mapping of the hMR gene suggests the PHA
locus should reside on chromosome 4.

Cloning and expression of functional hMR has
provided unexpected insight and should stimulate new
interest into the mechanisms underlying physiologic
20  complexity, and allow the development and testing of
new models for the coordinate regulation of gene
networks.

IV. J. DETAILED DESCRIPTION OF FIGURES
REFERRED TO IN EXPERIMENTAL SECTION IV
25  Figure IV-1 (A), (B), (C) and (D).

Isolation of a genomic sequence related to the
hGR gene. (A) High-stringency Southern analysis of
human placenta DNA digested with the indicated
restriction endonucleases. hGR cDNA (hGR1.2) was used
30  as a probe. Sizes of lambda DNA fragment markers (in
kilobase pairs) prepared by HindIII digestion are
indicated next to the autoradiogram. (B)
Low-stringency Southern analysis. The 2.5-kbp band
bracketed by asterisks in the HindIII lane was the
35  sequence targeted for direct genomic cloning. (C)
Isolation of this genomic sequence in a clone

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designated lambda HGH is demonstrated by its use as a
probe on a similar Southern blot.   The lambda HGH
genomic fragment contains the hybridizing inter..al
_EcoRI_ fragment isolated from this cloning.   (D) The
5   intron-exon structure of the lambda HGH genomic
fragment and its homology with hGR.   The hGR-related
exon found within lambda HGH is boxed in black with its
predicated amino acid sequence.   Conserved cysteine
residues are indicated with white dots.   Portions of
10   intron sequence with consensus splice donor and
acceptor sites underlined are shown flanking the exon.
Nucleotide homology with the hGR is shown underneath.
Nucleotide numbers for hGR are from Figure I-2(1) and
I-2(2), discussed in Experimental Section I; also _see_
15   Hollenberg, et al. (1985) (ref. 8) for publication of
the study used herein as Experimental Section I.   For
Southern analysis, we digested DNA from human term
placenta with restriction endonucleases, and products
were separated on a 0.8 percent agarose gel.   The DNA's
20   were transferred to nitrocellulose paper and hybridized
under either stringent or nonstringent conditions.
Stringent hybridization was performed with 50 percent
formamide, 5 x SSPE (NaCl, $NaH_2PO_4$, EDTA, pH 7.4), 1 x
Denhardt's, 0.1 percent SDS, salmon sperm DNA at 100
25   microgram/ml, and probe ($10^6$ cpm/ml) at 42°C.   For
nonstringent hybridization, 35 percent rather than 50
percent formamide was used.   Washing conditions
consisted of 0.1 x SSC (standard saline citrate) with
0.1 percent SDS at 60°C for stringent analyses and 2 x
30   SSC with 0.1 percent SDS at 55°C for nonstringent
filters.   Washing conditions with the 338-bp inset from
lambda HGH as probe were modified to 2 x SSC with 0.1
percent SDS at 68°C.   For isolation of lambda HGH,
human placenta DNA (300 microgram) was digested with
35   _HindIII_ and size-fractionated on a 1 percent
low-melting agarose gel (Seaplaque, FMC).   The gel was

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sliced in 0.5-cm strips, and the DNA was purified by
phenol extraction and ethanol precipitation. DNA (2
microgram) from the fraction corresponding in size to
the band bracketed by asterisks in (B) was repaired
5     with Klenow DNA polymerase for EcoRI linker addition.
After digestion with EcoRI and removal of excess
linkers on a Sepharose 4B column, this DNA was ligated
to EcoRI-digested lambda gt10 DNA and packaged in vitro
(lambda arms and extracts-from Vector Cloning Systems,
10    San Diego, California). About 4 x 10$^5$ independent
recombinants were screened under conditions identical
to those used for the nonstringent Southern analysis to
obtain lambda hGH.

Figure IV-2 (A) and (B).

15            Nucleotide sequence and primary amino acid
structure of human mineralocorticoid receptor. (A)
Composite structure of hMR aligned with a line diagram
of some restriction endonuclease cleavage sites (EcoRI
sites shown at nucleotides I and 5823 and derived from
20    linkers). The composite was assembled from two
overlapping lambda gt10 clones, lambda hk10 and lambda
hk2. Parentheses in the line diagram of lambda hk2
indicate a 351-bp deletion. The hatched box indicates
predicted coding sequence with initiator and
25    termination codons indicated. (B) Complete nucleotide
sequence of hMR and its predicted primary amino acid
sequence. Underlined are a 5' in-frame termination
codon upstream of the predicted initiator methionine
and four potential polyadenylation sites (AATAAA).
30    Human kidney lambda gt10 libraries (18) were screened
with the insert from lambda HGH under the same
conditions described for Southern analysis under
high-stringency conditions with this probe. Overlapping
deletions of each cDNA were obtained (36) by the
35    Cyclone rapid deletion subcloning method (International
Biotechnologies). Deletion clones were

sequenced by the dideoxy procedure (37), and any gaps
or ambiguities were resolved by the chemical cleavage
method (38).  DNA sequences were compiled and analyzed
by the programs of Devereux, et al. (39) and Staden
5    (40).
Figure IV-3.
        Amino acid homology of mineralocorticoid
receptor with glucocorticoid receptor.  The primary
amino acid sequence of hMR has been aligned with that
10   of hGR for maximum homology by introducing gaps as
indicated by dots.  Numbers were taken from Figs.
IV-2(B)-1 and IV-2(B)-2 for hMR and from Figure I-2(1)
and I-2(2) for hGR.  No significant homology was found
upstream of the region shown. Vertical lines indicate
15   identical amino acid residues. Arrows show putative
boundaries of the DNA-binding (DNA) and steroid-binding
(Steroid) domains.  The amino-terminal border of the
DNA-binding domain was arbitrarily defined by the first
conserved cysteine residue while the carboxyl-terminal
20   limit was chosen on the basis of mutagenesis studies
which indicated sequences necessary for DNA-binding and
transcriptional activation (15).  Several conserved
basic residues that follow the DNA binding domain may
also be important for these functions.  The limits of
25   the steroid-binding domain, while defined by the region
of amino acid homology, are also consistent with
mutational analysis. Single letter abbreviations for
the amino acid residues are: A, Ala; C, Cys; D, Asp; E,
Glu; F, Phe, G, Gly; H, His; I, Ile; K, Lys; L, Leu; M,
30   Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V,
Val; W, Trp; and Y, Tyr.
Figure IV-4 (A), (B), (C) and (D).
        Steroid-binding properties of expressed hMR.
(A) Structure of pRShMR, the hMR expression plasmid
35   (41).  (B) Scatchard analysis of tritiated aldosterone
binding in extracts prepared from pRShMR-transfected

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COS cells.  Each point was assayed in triplicate with
100 micrograms of extract protein in a 200-microliter
incubation at 0°C for 2.5 hours.  The nonspecific
5   binding determined with a 500-fold excess of unlabeled
aldosterone was approximately 20 percent of total
counts.  No specific binding was seen in
mock-transfected cells.  (C and D) Competition of
unlabeled steroids for binding with 5 nM
10  [3H]aldosterone in transfected COS cell extracts.  The
results of two independent trials representative of
these competition experiments are shown.  Cold
competitor was present in 1-, 10-, or 100-fold molar
excess.  The value for 100 percent binding was
15  determined by subtracting the number of counts per
minute bound in the presence of 1000-fold excess of
unlabeled aldosterone from the counts bound in the
absence of competitor.  Abbreviations:  Aldo,
aldosterone, Doc, deoxycorticosterone; Dex,
20  desamethasone; Spiro, sprionolactone; E21, 17
beta-estradiol; CS, corticosterone; HC, hydrocortisone;
and Prog, progesterone.  Subconfluent COS cells were
transfected by the DEAE-dextran method (42) with 10
micrograms of pRShMR per dish.  Cells were maintained
25  for 2 days in DMEM (Dulbecco's modification of Eagle's
minimum essential medium) with 5 percent
charcoal-treated fetal calf serum, then harvested [in
40 mM tris-HCl (pH 7.8), 10 mM NaCl, 1 mM EDTA, 10 mM
$Na_2MoO_4$, 5 mM dithiothreitol, antipain (5
30  microgram/ml), leupeptin (5 microgram/ml), and 500
microM phenylmethylsulfonyl fluoride.  After
centrifugation at 15,000 x g for 10 minutes, extracts
were adjusted to 100 mM NaCl and 5 percent glycerol
before binding.  Labeling reactions with
35  [3H]aldosterone (specific activity 78 Ci/mmol,
Amersham) were incubated for 2.5 hours at 0°C in a
total volume of 200 microliters, and then for 10

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minutes with 20 microliters of 50 percent
dextran-coated charcoal (10:1 activated
charcoal:dextran). After centrifugation at 15,000 x g
for 2 minutes at 4°C, tritium in supernatant was
5   quantified by liquid scintillation spectrophotometry.
_Figure IV-5 (A), (B) and (C)._

Transcriptional activation of MMTV LTR by hMR
and hGR expression plasmids in transfected CVI cells.
(A) Structure of GMCAT. This plasmid was cotransfected
10  with the steroid receptors as a reporter gene for
hormone-dependent transcriptional activation (see
Experimental Section II). (B) Differential CAT enzyme
activity found after hMr or hGR transfection with
normal serum. Transfected cells were maintained in
15  DMEM with 5 percent fetal calf serum. Serum was
treated with charcoal to eliminate free steroids in
subsequent experiments so that the effects of exogenous
steroids could be determined. (C) Differential
induction of CAT activity by aldosterone or
20  dexamethasone in cells transfected with hMR or hGR. CVI
cells were cotransfected with 10 micrograms of either
pRSVgal (control), pRShMR, or pRShGR alpha and 10
micrograms of the reporter GMCAT and cultured in the
absence (-) or presence of 10 nM aldosterone (A) or 10
25  nM dexamethasone (D). AC, 3-acetylchloramphenicol; C,
chloramphenicol. Two days after transfection by
calcium phosphate coprecipitation (43), extracts were
prepared for CAT assay (44). The assays were incubated
for 6 hours with 50 micrograms of protein extract.
30  _Figure IV-6._

Northern analysis of mineralocorticoid
receptor mRNA's in rat tissues. The 1270-bp EcoRI
fragment (1770 to 3040) from lambda hk10 was used as a
probe for the expression of homologous mRNA's in rat.
35  Ten micrograms of poly(A)$^+$ mRNA was used in all lanes.
Migration of ribosomal RNA's (28S and 18S) are

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indicated for size markers.  After hybridization under
stringent conditions, the filter was washed twice for
30 minutes each time in 2 x SSC with 0.1 percent SDS at
68°C.

5    Figure IV-7.

Chromosomal localization of hMR gene by
Southern analysis of microcell hybrids.  The
construction and characterization of these hybrids has
been previously described (32).  The human chromosome
10   content of each is as follows:  HDm-4A (chromosome 20),
HDm-5 (chromosome 14 and an unspecified E group
chromosome), HDm-9 (chromosomes 20, 14, and 21), HDm-15
(chromosomes 21, 11, and 4), HDm-20 (chromosomes 7 and
14), and HDm-1132B (chromosome 4 only).  Human (HeLa)
15   and mouse (3T#) control DNA samples are also shown.
Genomic DNA from microcell lines (10 micrograms) was
digested with EcoRI and subjected to electrophoresis
through a 1.0 percent agarose gel, transferred to a
nylon membrane (Nytran, Schleicher & Schuell), and
20   hybridized with a hMR cDNA probe under high-stringency
conditions (Fig. IV-1(A)).  Radioactive probe was
synthesized by the Klenow fragment of Escherichia coli
DNA polymerase from two randomly primed (45) hMR cDNA
templates (the 1000- and 800-bp EcoRI fragments of
25   lambda hk2).  The sizes of HindIII-digested lambda DNA
fragments are indicated next to the autoradiogram.
Figure IV-8.

Schematic amino acid comparisons of the hGR,
hMR, and rPR structures.  Primary amino acid sequences
30   have been aligned schematically with the percentage
amino acid identity indicated for each region of
homology in the intervals between dotted lines.  The
amino acid position of each domain boundary is shown
for each receptor.  N and C represent the amino and
35   carboxyl termini, respectively.  Cys corresponds to the
cysteine-rich region encoding the putative DNA-binding

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domain while Steroid (cortisol, aldosterone, or
progesterone) designates the steroid-binding domain.
The immunogenic region (IMM) of the hGR is also
5   indicated. Amino acid residue numbers are taken from
(see Experimental Section I) for hGR, Loosfelt, et al.
(11) for rPR, and from our data for hMR.

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41.　　Construction of pRShMR.  The 3.75-kb insert
　　　　from lambda hk10 was ligated into the EcoRI
　　　　site of pGEN4 (Promega) oriented with the mp18
30　　　 polylinker adjacent to the 5' end of hMR
　　　　coding sequence.  Digestion of this plasmid
　　　　(phk10) with HindIII generated at HindIII
　　　　fragment spanning the polylinker site to the
　　　　hMR site at position 3562; this fragment was
35　　　 isolated and the ends were repaired with the
　　　　Klenow fragment of DNA polymerase I. Plasmid
　　　　pRSVCAT [C.M. Gorman, G.T. Merlino, M.C.

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Sillingham, I. Pastan, B.H. Howard, Proc. Natl. Acad. Sci., USA, 79:6777 (1982)] was digested with HindIII and HpaI, and the HindIII-HpaI fragment containing pBR322

5  sequences, the RSV LTR, and the SV40 polyadenylation site was also repaired. Ligation of the hMR fragment to the fragment from pRSVCAT yielded a vector which, in the correct orientation, has hMR coding sequence

10  driven by the RSV promoter. Sites bracketed in Fig. IV-4(A) were lost in this cloning step. To improve translational efficiency several upstream initiation and termination codons in the 5'-untranslated region were deleted by

15  digesting the vector with AccI to remove an ¯200-bp sequence from the mpl8 polylinker to position 188 in the hMR 5'-UT region. Finally, an NdeI-linkered SV40 origin of replication was introduced into the NdeI site

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EXPERIMENTAL SECTION V

IDENTIFICATION OF A NEW CLASS OF

STEROID HORMONE RECEPTORS

V. A. INTRODUCTION

5         The gonads and adrenal glands produce a large
variety of steroids classified into five major groups
which include the estrogens, progestins, androgens,
glucocorticoids and mineralocorticoids. Gonadal

10 steroids control the differentiation and growth of the
reproductive system, induce and maintain sexual
characteristics and modulate reproductive behavior.
Similarly, adrenal steroids influence differentiation
in addition to their vital roles as metabolic

15 regulators. Despite this wide range of physiological
actions, the effects of each steroid rest primarily
upon the specific cognate receptors which it binds, and
therefore steroid hormone action might be more
precisely classified according to the receptors that

20 mediate their biologic action. The successful cloning,
sequencing and expression of the human glucocorticoid
receptor (hGR) cDNA (see Experimental Section I, which
was published as ref. 1), soon followed by those
encoding the estrogen[2,3] (hER), progesterone[4] (hPR),

25 and mineralocorticoid (hMR receptors) (see Experimental
Section IV, which was published as ref. 5), plus
homologues from various species[6-11], provide the first
opportunities to study receptor structure and the
molecular mechanisms by which these molecules modulate

30 gene expression. Sequence comparison and mutational
analysis of these proteins reveal structural features
common to all classes of steroid hormone receptors (see
Experimental Section II which was published as ref. 12;
also see refs. 13 and 14). In particular, the

35 receptors share a highly conserved cysteine rich
region, now referred to as the DNA-tau domain, that
contains all the information required for both

DNA-binding and trans-activation functions of the
glucocorticoid receptor[15,16]. The presence of a common
segment between receptors provides the possibility of
scanning the genome for related gene products. For
example, MR cDNA was isolated by using the hGR as a
hybridization probe (see Experimental Section IV, which
has been published as ref. 5). One way that molecular
biology can advance our understanding of health and
10  human disease and the physiology that governs these
events is through the identification of new hormone
response systems. In this study, using the highly
conserved DNA-tau region of the human estrogen receptor
cDNA as a hybridization probe, we have isolated two
15  cDNA clones encoding polypeptides that comprise the
structural features of the steroid hormone receptors.

### V. B. cDNA CLONES FOR RECEPTOR hERR1

One approach to search for unrecognized
hormone response systems is to systematically employ
20  reduced stringency hybridization to screen recombinant
DNA libraries for novel hormone receptors. The DNA-tau
segment of the estrogen receptor was used to initiate
these studies. Analysis of a lambda gt10 human testis
cDNA library identified 3 positives at a frequency of
25  one clone per $3 \times 10^5$ recombinant phages. Nucleotide
sequence analysis revealed that two of these clones
actually encode the estrogen receptor while the third
one, spanning 2.0 kilobases and named lambda hT16,
showed only partial sequence homology. In turn, this
30  clone was used to screen human fetal kidney and adult
heart cDNA libraries, resulting in the identification
of 3 additional clones. Both clones from the kidney
library, lambda hKE4 and lambda KA1, represent the same
gene product as lambda hT16 while the cardiac clone,
35  lambda hH3, is only partially related. The composite
sequence of the three cDNA's sharing identical
sequences, herein referred to as hERR1, is shown in

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Figures V-1(B)-1 and V-1(B)-2. Assuming a poly(A) tail
of ~150-200 nucleotides[17], this sequence (~2430 nt)
must be nearly full length. The cDNA insert from lamb-
da hKA1 contains nucleotide 179 to 2430 while lambda

5   hKE4 represent a rare messenger RNA splicing error with
deletion of exon 2 and insertion of intron sequences.
The exon/intron boundaries suggested by lambda hKE4
were confirmed by cloning and partially sequencing the
genomic fragments encoding this gene (data not shown).

10  The sequence surrounding the first ATG agrees with the
concensus described by Kozak[18] for a translation
initiation site. An open reading frame of 521 amino
acids predicting a polypeptide of Mr 57300 is flanked
by a 775 nucleotide 3'-untranslated region.

15          V. C. cDNA CLONE FOR RECEPTOR hERR2
            The characterization of clone lambda hH3 re-
veals it to encode a unique polypeptide highly related
to hERR1. Figures V-2(B)-1 and V-2(B)-2 show the 2153
nucleotide sequence of lambda hH3 and the primary

20  structure of the protein product designated hERR2. The
translation initiation site was assigned to the
methionine codon corresponding to nucleotides 100-102
because this is the first ATG triplet that appears
downstream from an in-frame terminator TGA (nucleotides

25  31-33). An open reading frame containing 433 amino
acids encodes a polypeptide of Mr 47600 and is followed
by a 3'-untranslated region of 752 nucleotides.
            V. D. CHARACTERIZATION OF hERR1 AND hERR2
            As mentioned earlier, steroid hormone

30  receptors are composed of distinct functional domains
that can be identified by sequence analysis[14]. The
predicted hERR1 and hERR2 polypeptides contain the
expected domain features of steroid receptors. Amino
acid comparison between hERR1 and hERR2 shows that

35  these two proteins have divergent amino termini and
that no homology can be detected with other classes of

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receptor within this region (data now shown). This
finding is in agreement with previous comparison
studies (see Experimental Section IV; also see refs.
5,8,10,14) which showed this region to be hypervariable
in sequence. Alignment of the carboxy-terminal region
of hERR1, hERR2, hER and hGR (Fig. V-3) shows that the
highest degree of homology between these proteins is
found in a cysteine-rich region of 66 amino acids,
corresponding to the DNA-tau domain (see Experimental
Section II; also see ref. 15) of the steroid hormone
receptors, located between amino acid 175 and 240 of
hERR1. There is a 91% amino acid identity in the
comparison of hERR1 with hERR2, 68% with hER and 56%
with hGR. The positions of the 9 cysteine residues are
strictly conserved but the absence of a histidine
residue at position 206 of hERR1 and position 134 of
hERR2 marks a major difference with the previously
described steroid hormone receptors. It was originally
thought that this histidine residue might be involved
with the conserved cysteine residues in the formation
of a DNA-binding finger. The recent demonstration that
this histidine residue is also absent in the
corresponding amino acid sequence of the vitamin D
receptor[19], another member of the ligand-binding
transactivation factor superfamily, suggests that $Zn^{2+}$
atoms interact exclusively with cysteine residues in
order to coordinate the formation of the proposed
DNA-binding fingers present in those proteins. The
putative steroid binding domain, positioned between
amino acid 295 and 521 of hERR1, shows 63% identity
when compared to hERR2, 36% to hER and 28% to hGR.

### V. E. TISSUE DISTRIBUTION OF mRNA's FOR hERR1 AND hERR2

Steroid receptors are expressed in
characteristic tissue specific patterns that directly
correlate to their primary physiologic effects.

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Perhaps, the distribution of these putative receptors would provide a clue to their hidden identity. Accordingly, total RNA isolated from a variety of rat and human tissues was fractionated on

5   formaldehyde-agarose gel electrophoresis and transferred to nitrocellulose filters. Using lambda hKA1 as a probe, a 2.6 Kb mRNA encoding hERR1 was detected in all rat and human tissues analyzed, with surprisingly high levels in the cerebellum and

10  hippocampus and the lowest levels seen in the liver, lungs, seminal vesicles and spleen (Fig. V-1(A)). Thus, it appears that the hERR1 gene is widely and abundantly expressed, although present in much higher levels in the rat central nervous system. In contrast

15  to the hERR1 mRNA expression pattern, the distribution of the mRNA encoding the hERR2 protein is restricted to a few specific tissues where very low levels of mRNA can be detected (Fig. V-4(B)). Using a probe derived from the clone lambda hH3, a 4.8 Kb mRNA was detected

20  in kidney, heart, testis, hypothalamus, hippocampus, cerebellum and rat prostate. However, no hybridization could be detected in the human placenta or prostate. Considering the difference in exposure time and the resulting signal intensity, levels of hERR2 mRNA are

25  approximately 10 to 100-fold lower than that of hERR1.

### V. F. HOMOLOGY BETWEEN hERR1 AND hERR2

Prior studies indicate that the degree of homology of the ligand binding domains between the steroid hormone receptors reflects the structural

30  relatedness of their ligands. For example the hGR, hMR and hPR, which show 56% identity in their ligand binding domains (see Experimental Section IV), bind closely related hormones. Indeed the hMR binds glucocorticoids with an affinity equal to that of

35  aldosterone, and also binds progesterone with relatively high affinity (see Experimental Section IV).

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In the case of the hERR gene products, amino acid
sequence homology reveals a relatively more distinct
relationship with hER, 70% in the DNA-tau region and
36% in the steroid binding region (Fig. V-5).  These
5   levels of homology are lower than those observed
between hGR, hMR and hPR and thus predict that the
putative hERR proteins interact with a class of steroid
hormones distinct from the estrogens.  However, the
homology between hERR1 and hERR2 suggests that they are
10  receptors for either a single or two closely related
steroid metabolites.  Preliminary steroid binding
studies using the products of *in vitro* translation of
capped SP6 RNA produced from hERR1 and hERR2 coding
sequences or expression of the two cDNA's in COS-1
15  cells (see Experimental Sections IV and II) have failed
to demonstrate binding of any major classes of
steroids, including estrogens and androgens.

### V. G. CONCLUSION

The tissue distribution of hERR1 and hERR2
20  mRNA's expression suggests that each putative receptor
will control distinct biological functions.  How might
the functions of these steroid hormone receptors have
been overlooked?  Most likely many of their activities
have erroneously been attributed to other receptors
25  with differences being classified as atypical effect.
The recent identification of neuronal steroids[20,21]
provides evidence for new steroid hormones with
possible paracrine actions within the brain.  Such
systems could have easily escaped previous
30  physiological detection.  Thus, the isolation two novel
steroid hormone receptor cDNA's marks the first step
toward identifying a new hormone response system.

### V. H. DETAILED DESCRIPTION OF FIGURES
### REFERRED TO IN EXPERIMENTAL SECTION V

35  Figure V-1 (A) and (B).

Restriction map (A) and DNA sequence and

predicted amino-acid sequence (B) of hERR1.  A, The
composite cDNA for hERR1 is represented at the top,
with noncoding (thin line) and coding (stippled
portion) sequences indicated.  Common 6-nucleotide
5    restriction enzyme sites are drawn above the linear
map.  Overlapping cDNA inserts used to determine the
sequence are shown.  The wavy line near the 5' end of
lambda hKE4 indicates divergent sequence.  B,
Nucleotide sequence of the composite hERR1 cDNA with
10   the deduced amino acids given above the long open
reading frame.
Figure V-1 (A) and (B) Methods.
           The clone lambda hT16 was isolated from a
human testis lambda gt10 cDNA library (Clonetech) using
15   a nick-translated[22] 446-bp BclI-BamHI fragment isolated
from pER945, a linker-scanning mutant[12] derived from
the hER cDNA (V. Giguere and R.M. Evans, unpublished
data).  The hybridization mixture contained 35%
formamide, 1X Denhardt's, 5X SSPE, 0.1% sodium dodecyl
20   sulfate (SDS), 100 micrograms ml$^{-1}$ denatured salmon
sperm DNA and $10^6$ c.p.m. ml$^{-1}$ of $^{32}$P-labeled BclI-BamHI
fragment ($>10^8$ cpm/microgram).  Duplicate
nitrocellulose filters were hybridized at 42ºC for 16
h, washed three times for 20 min each in 2X SSC, 0.1%
25   SDS (1 X SSC=150 mM Nacl, 15 mM sodium citrate) at 55ºC
and autoradiographed at -70ºC with an intensifying
screen.  The clones lambda hKE4 and lambda hKA1 were
isolated from a human kidney lambda gt10 cDNA library[23]
using the nick-translated insert from lambda hT16.  For
30   this screening, the hybridization mixture was modified
to 50% formamide and washing conditions to 2X SSC with
0.1% SDS at 68ºC.  The cDNA clones were digested with a
number of restriction enzymes and the resulting
fragments were subcloned in both orientations into the
35   M13 sequencing vectors mp18 and mp19 and sequenced by
the dideoxy procedure[24], and any gap or ambiguities

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were resolved by the chemical cleavage method[25]. DNA
sequences were compiled and analyzed by the programs of
Devereux, et al.[26] and Staden[27].

Figure V-2 (A) and (B).

5          Restriction map (A) and DNA sequence and
predicted amino-acid sequence (B) of hERR2. A,
Schematic representation of hERR2 cDNA; some common
restriction enzyme sites are indicated. The stippled
box represents the predicted open reading frame. B,

10  The complete nucleotide sequence of lambda hH3 is shown
with the predicted amino acid sequence given above the
long open reading frame. A short open reading frame in
the 5' untranslated region is shown in bold type.

Figure V-2 (A) and (B) Methods.

15          The clone lambda hH3 was isolated from a human
heart lambda gt11 cDNA library (gift from Dr. L.A.
Leinwand, Albert Einstein Col. of Med.) using a
nick-translated 700-bp EcoRI-SmaI fragment representing
the 5' portion of lambda hKA1. Hybridization and

20  washing conditions and sequencing strategies were as
described in Fig. V-1(A) and (B) for the screening of
the human kidney library.

Figure V-3.

          Amino acid sequence comparison between the

25  carboxy-terminal regions of hERR1, hERR2, the human
oestrogen and glucocorticoid receptors. The four amino
acid sequences were aligned for maximum homology by
introducing gaps as indicated by hyphens. Numbers were
taken from Figs. V-1(B)-1 and V-1(B)-2 and V-2(B)-1 and

30  V-2(B)-2 for hERR1 and hERR2, from Figure I-2 for hGR
and Green, et al.[2] for hER. Amino acid residues
matched in at least three of the polypeptides are
boxed. The asterisk above residue 206 of hERR1
indicates the position of the histidine residue present

35  in the hER sequence but absent in both hERR1 and hERR2
sequences.

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<u>Figure V-4 (A) and (B).</u>

Northern blot hybridization analysis of hERR1
(A) and hERR2 (B) mRNA's in rat and human tissues.
<u>Figure V-4 (A) and (B) Methods.</u>

5      Total RNA was isolated from various tissues
using guanidine tiocyanate[28], separated on 1%
agarose-formaldehyde gel, transferred to
nitrocellulose, and hybridized under stringent
conditions using a nick-translated <u>Eco</u>RI-<u>Sma</u>I fragment

10     from lambda hKA1 (a) and a nick-translated 1192-bp
<u>Eco</u>RI-<u>Hind</u>III fragment from lambda hH3 (b).  Twenty
micrograms of total RNA was used in all lanes.
Migration of ribosomal RNA's (28S and 18S) are
indicated for size markers.  The nitrocellulose filters

15     were autoradiographed at -70°C with an intensifying
screen for 24 h (a) and 6 days (b).  Apparent
difference in migration rate of the mRNA in (a) is an
artifact from the gel.
<u>Figure V-5.</u>

20     Schematic amino acid comparisons between hERR1
and hERR2, hER, hGR and human thyroid hormone receptor
(hT$_3$R beta).  Amino acid sequences have been aligned
schematically according to the functional domain
structure of the steroid and thyroid hormone receptors

25     superfamily[14].  The percentage of amino acid identity
of each receptor with hERR1 is indicated inside each
domain.  The amino acid position of each domain
boundary is shown for each receptor.

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### EXPERIMENTAL SECTION VI
### A c-erb-A BINDING SITE IN THE RAT GROWTH HORMONE GENE MEDIATES TRANSACTION BY THYROID HORMONE
### VI. A. INTRODUCTION

5        The substance 1,3,3-triiodothyronine ($T_3$) stimulates growth hormone gene transcription in rat pituitary tumor cells[1-4]. This stimulation is thought to be mediated by the binding of nuclear $T_3$ receptors to regulatory elements 5' to the transcriptional start

10    site[5-8]. Understanding of the mechanism by which thyroid hormone activates gene transcription has been limited by failure to purify nuclear $T_3$ receptors because of their low abundance, and by the absence of defined $T_3$ receptor-DNA binding sites affecting $T_3$

15    regulation. Recently, human and avian c-erb-A gene products have been shown to bind thyroid hormone with high affinity (see Experimental Section III, which has been published as ref. 10; also see ref 9) and to have a molecular weight and nuclear association

20    characteristic of the thyroid hormone receptor. In the present study, we describe the development of an avidin-biotin complex DNA-binding assay which can detect specific, high-affinity binding of rat pituitary cell $T_3$ receptors to the sequence 5'CAGGGACGTGACCGCA3',

25    located 164 base pairs 5' to the transcriptional start site of the rat growth hormone gene. An oligonucleotide containing this sequence transferred $T_3$ regulation to the herpes simplex virus thymidine kinase promoter in transfected rat pituitary GC2 cells, and

30    specifically bound an in vitro translation product of the human placental c-erb-A gene. The data provide supporting evidence that human c-erb-A gene mediates the transcriptional effects of $T_3$ and also that GC2 cell nuclear extracts contain additional factors that

35    modify the binding of pituitary $T_3$ receptors to the rat growth hormone gene $T_3$ response element.

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### VI. B. CHARACTERIZATION OF RAT GROWTH HORMONE
### DNA SEQUENCES NECESSARY FOR T3 REGULATION
### (a) RAT GH GENE DELETION ASSAYS

5        To identify the cis-active element in the
growth hormone (GH) 5' flanking genomic sequence
necessary for $T_3$ regulation we used a series of
5'-deleted fragments of the rat GH gene, fused to the
bacterial chloramphenicol acetyltransferase (CAT) gene
and transfected into rat pituitary GC2 cells (Fig.
10   VI-1(A)). A 5' deletion to -235 to the transcriptional
start (CAP) site transferred regulation to $T_3$
(4.6-fold, Fig. VI-1(A)), equivalent to constructions
containing 1.7 kilobases (kb) or 307 base-pairs (bp) of
5'-flanking rat GH information (data not whown), in
15   agreement with previous studies[5,6]. (Nucleotide
positions are numbered relative to the CAP site,
negative position numbers being 5' to it.) Deletions
containing less than 235bp of 5'-flanking GH
information could not be assayed for $T_3$ induction
20   because the levels of CAT expression in the absence of
$T_3$ were not significantly above background. To
overcome this problem, a rat prolactin enhancer
element[11] was fused proximal to fragments of the rat GH
gene containing 181 and 110 bp of 5' flanking
25   information. The 5'-deleted fragment extending to
position -181 gave 2.6-fold induction by $T_3$ and further
deletion to position -107 from the CAP site abolished
$T_3$ regulation (Fig. VI-1(A)).

### (b) CAT ENZYMATIC ASSAY

30       To assay 3' deletions, fragments of the rat Gh
gene were fused to the herpes simplex virus thymidine
kinase (HSV tk) promoter. A fragment of the Gh gene
extending from position -235 to position -45 from the
CAP site produced 2.5- and 2.3-fold stimulations of CAT
35   activity when fused to the tk promoter in native and
inverse orientation, respectively (Fig. VI-1(B)). A

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90-bp fragment extending from positions 235 to 145 bp
from the GH CAP site transferred $T_3$ regulation even
more efficiently than the 235-45 bp fragment, and
suggests that a negative $T_3$ regulatory element might be
5    located between 45 and 145 bp from the CAP site (Fig.
1(A)).  CAT messenger RNA was analyzed by a primer
extension technique [12] to determine whether or not the
$T_3$-dependent stimulation of CAT activity which was
observed in cells that had been transfected with
10   plasmids containing the tk promoter resulted from an
increase in appropriately initiated transcripts.  The
235-45 bp GH fragment fused to the tk promoter gave an
increase of about 4-fold of correctly initiated CAT
mRNA in the presence of $T_3$ (Fig. VI-1(C)), a result
15   consistent with the observed CAT activity measurements.
(c) AVIDIN-BIOTIN COMPLEX DNA-BINDING ASSAY (ABCD
ASSAY)

          To define further the sequences needed for $T_3$
regulation, it was necessary to document specific
20   binding of nuclear $T_3$ receptors to the growth hormone
$T_3$ regulatory element.  Because attempts to map the $T_3$
receptor binding site by gel shift and footprint- ing
assays were initially unsuccessful, a new assay to
detect specific binding was devised, the avidin-biotin
25   complex DNA-binding (ABCD) assay.  Double-stranded
oligonucleotides were prepared containing the 5'
flanking region of the GH gene necessary for $T_3$
regulation, with biotin-11-dUTP at various positions,
as shown in Fig. VI-2(A).  Initially a 77-bp
30   oligonucleotide (G209-146), containing the genomic
growth hormone sequence from positions -209 to -146,
was used.  $T_3$ receptors from GC2 nuclear extracts were
labeled to high specific activity with $^{125}I-T_3$ and
incubated with this biotinylated oligonucleotide.
35   Protein-DNA complexes were precipitated from solution
after the binding reaction, using streptavidin

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conjugated to agarose beads. It can be seen in Fig.
VI-2(B) that probe G209-146 resulted in the precipita-
tion of 6,900 c.p.m. of $^{125}I$-$T_3$ activity. This
represents the binding of about 3.2 fmol of $T_3$ receptor

5   and accounts for ~40% of the total $T_3$ receptor present
in the binding reaction. Precipitation of
$^{125}I$-$T_3$-labeled receptors was probe-dependent; <15% of
total precipitated $^{125}I$-$T_3$ radioactivity was recovered
in the absence of G209-146. Addition of a 100-fold

10  molar excess of unlabeled $T_3$ reduced the precipitated
$^{125}I$-$T_3$ to background levels (Fig. VI-2(B)), indicating
that the $T_3$ binding protein that was being precipitated
by the probe was present in limited amounts. The
equilibrium binding constant for the $T_3$ receptor-DNA

15  complex was estimated to be 1.4 x $10^{-9}M$ (data now
shown).

To investigate whether the precipitation of
labeled $T_3$ receptors by G209-146 was dependent on
specific rat GH sequences, a series of biotinylated

20  probes were prepared that had no apparent sequence
similarity to the growth hormone enhancer but which
were the same length as G209-146 and contained the same
number of biotin-11-dUTP residues. As shown in Fig.
VI-2(B), the addition of 100 fmol of each probe to GC2

25  nuclear extracts gave no measurable precipitation of
$^{125}I$-$T_3$. This indicates that the precipitation of
$^{125}I$-$T_3$ by G209-146 is dependent on the rat GH sequence
contained in the probe.

(d) FOOTPRINTING ANALYSIS

30      Although early attempts to localize a $T_3$
receptor binding site using conventional footprinting
techniques were unsuccessful, variation of buffering
conditions in the ABCD binding assay suggested that a
footprint might be achieved with crude nuclear extracts

35  if salt and pH conditions for DNA binding were optimal
(data not shown). End-labeled fragments of the GH
enhancer were incubated with GC2 nuclear extracts and

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digested with DNase I.  PAGE analysis of the digest
under denaturing conditions (Fig. VI-3) gave two
footprints, described previously[13,14]; one of these is
shown, together with a 16-bp protected region in the
5  antisense strand between nucleotides -179 and -164.
This sequence, which in the sense strand corresponds to
5' CAGGGACGTGACCGCA 3', is contained in the
oligonucleotide probe shown to specifically bind $T_3$
receptors, and corresponds in position to a previously
10  identified $T_3$-dependent DNase-I-hypersensitive site[19].
A clear footprint could not be detected in the sense
strand itself, mainly because of incomplete digestion
by DNase I in this G-rich region.

VI. C. FUNCTIONALITY OF $T_3$ OLIGONUCLEOTIDE
15      To examine the function of this sequence in $T_3$
regulation of the GH gene, site-directed mutagenesis
was used to delete 11 bp of the footprint from the
wild-type enhancer (mutant G delta 166/177).  Addition
of $T_3$ to cells transfected with mutant G delta 166/177
20  fused to the tk promoter had no effect on the amount of
CAT expression, although the wild-type enhancer
stimulated CAT expression ninefold (Fig. VI-1(B)).
Thus, removal of the putative $T_3$ receptor binding
region, identified by oligonucleotide and DNAse I
25  binding assays, abolished the ability of the GH
enhancer to confer $T_3$ regulation to the tk promoter.
        To demonstrate that the 16 base pairs from
positions -164 to -179 constituted a functional $T_3$
regulatory element, a double-stranded oligonucleotide
30  was prepared containing this sequence with seven and
six bases flanking the 5' and 3' ends respectively.
This oligonucleotide was inserted in its native
orientation proximal to the tk promoter (construction
G29TK, Fig. VI-1(B)).  CAT expression was stimulated
35  2.9-fold by $T_3$ in cells transfected with this
construction.  Insertion of three tandem repeats of

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this sequence (construction G293TK) resulted in a
five-fold stimulation by $T_3$.

The short oligonucleotide used for transfer of
$T_3$ regulation to the tk promoter (G186-158) also
specifically bound nuclear $T_3$ receptor binding site in
G209-146, but this was discounted using an
oligonucleotide containing the rat GH sequence from
nucleotide positions -177 to -235 that failed to bind
measurable amounts of $T_3$ receptors (data not shown).
Non-biotinylated G209-146 and G186-158 were also used
to compete for the binding of $T_3$ receptors with
biotinylated G209-146; the relative affinity of $T_3$
receptors for G209-146 was two- to three-fold higher
than G186-158 (data not shown). The apparent decrease
in affinity for the shorter probe could result from a
lack of bases to participate directly in the $T_3$
receptor binding reaction. This is unlikely, because
the limits of the DNase I footprint lie within the ends
of this probe. Alternatively, GC2 nuclear extracts
could contain other factors that stabilize the binding
of $T_3$ receptors to the longer probe.

### VI. D. SUMMARY AND CONCLUSIONS

These experiments demonstrated that 29 base
pairs of the GH gene, containing a 13-bp footprint
extending from position -164 to position -179 5' to the
CAP site, bound the $T_3$ receptor, were necessary for $T_3$
regulation of the rat GH enhancer, and could transfer
$T_3$ regulation to the tk promoter. To test whether
human c-erb-A also binds to this element, an in vivo
translation product was labeled with $^{35}$S-methionine
(see Experimental Section III). The product migrated
as a doublet of relative molecular mass ($M_r$) 48,000
(48K) and 52K on SDS gel electrophoresis and bound $T_3$
with a $k_d$ of $5 \times 10^{+11}$ (data not shown). The binding
of the human c-erb-A in vitro translation product to
the G209-146 and G186-158 oligonucleotide probes

containing the rat GH T$_3$ regulatory element is shown in
Fig. VI-4.  Both the long and short probes bound the in
vitro translation product significantly, but probes
5   lacking homology to the T$_3$ receptor binding site of The
GH gene, such as P-EGF, did not.  Based on the
estimated specific activity of [35S]methionine present
in the in vitro translation mixture, the binding
activity shown in Fig. VI-4 corresponds to 1-2 fmol of
10  erb-A protein.  Unlike GC2-cell nuclear extracts
containing 125I-T$_3$ receptors, the c-erb-A in vitro
translation product was bound to the same extent by the
long and short oligonucleotides.  Similar data were
obtained using an in vitro translation product of human
15  c-erb-A labeled with 125I-T$_3$ (data not shown).  These
results indicate that the c-erb-A gene product
specifically binds to the identical T$_3$ regulatory
sequence that is bound by T$_3$ receptors from GC2 nuclear
extracts.  They provide further evidence that the
20  function of the c-erb-A gene product is to mediate the
transcriptional effects of T$_3$.

        Flug, et al. (1987) recently reported that the
GH sequence between nucleotide positions -210 and -181
was essential for the full stimulatory effect of T$_3$ in
25  transiently transfected GC cells, and also pointed out
that this region possessed limited similarity to other
T$_3$-regulated genes.  Our experiments locate the T$_3$
receptor-DNA binding site between 164 and 177 bp from
the CAP site, and also confirm the functional
30  importance of the sequence between positions -210 and
-181 in T$_3$ regulation of the GH gene.  This could
reflect an increased affinity of the T$_3$ receptor for
fragments of the GH enhancer which contain this
upstream element in vivo, as we observed in the in
35  vitro DNA binding studies using crude nuclear extracts
as a source of T$_3$ receptor.  That the erb A in vitro
translation product binds comparably to G209-146 and

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G186-158 is consistent with the possibility that crude
GC2 nuclear extracts contain an additional factor(s)
that binds to the sequence between positions -210 and
-181 and stabilizes the binding of the T3 receptor to
5    its cognate binding site.  Cooperative interactions
between eukaryotic transcription factors is
well-established[16-18]; in some cases this reflects the
ability of one factor to alter the DNA-binding affinity
of another.  Such interactions could be important in
10   the tissue-specific regulation of thyroid hormone
action[5,6].  The ABCD binding assay used here should be
useful in addressing these questions, and it is also
potentially applicable to any DNA-binding protein that
can be selectively radiolabeled, either with a labeled
15   ligand, by chemical modification or by in vitro
translation with labeled amino acids.

VI. F. DETAILED DESCRIPTION OF FIGURES
REFERRED TO IN EXPERIMENTAL SECTION VI

Figure VI-1 (A), (B) and (C).

20          Thyroid hormone responsiveness of various gene
fusions containing rat GH 5'-flanking sequences.  A,
Responsiveness of 5' and 3' deletions of the rat GH
gene.  5'-deleted fragments of the rat GH gene were
fused to the CAT gene in a pSV2CAT-based vector[18], in
25   which the SV40 origin of replication and promoter were
removed.  These constructions were transfected into GC2
cells and assayed for responsiveness to T3.  Because of
low levels of basal expression of the nucleotide
position -107 to +8 and -181 to +8 fragments, the rat
30   prolactin enhancer (Prl)[11], which is not regulated by
T3, was placed proximal to these elements.  The
illustrated 3'-deleted fragments were fused to a tk
promoter fragment, extending from position -197 to
position +54 and placed proximal to the CAT gene in the
35   same vector.  B, Functional analysis of the putative T3
receptor binding site.  Mutant G delta 177/166 contains

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a deletion of 11 bases of the $T_3$ receptor binding site from 177-166 base pairs from the CAP site. Plasmids G29TK and G293TK contain the 28-bp region of the rat GH gene that binds the $T_3$ receptor in one and three

5   copies, respectively. The effect of $T_3$ was determined by dividing the percentage conversion of chloramphenicol in the presence of $10^{-9}M$ $T_3$ by the percentage conversion in the absence of $T_3$. Triplicate plates were maintained in 10% fetal calf serum stripped

10   of $T_3$ by ion exchange chromatography for two days before transfection with test plasmids using DEAE-dextran[11]. The cells were treated with hormone 24 h after transfection and assayed for CAT activity after 24 h of $T_3$ exposure. Error limits represent the

15   standard error of the mean. Each construction was assayed two to five times. $A_n$ represents SV40 polyadenylation sites. C, Messenger RNA transcription initiation site analysis. The diagram indicates the 33-nucleotide primer complimentary to nucleotides 67 to

20   89 of the CAT coding sequence used to determine the CAP site of transcripts of plasmids containing the tk promoter. GC cells were transfected with test plasmids and hormone treated as described for the experiments presented in panels a and b. Primer extension analysis

25   was performed on 50 micrograms total RNA. Lanes A and B represent the extension product from cells transfected with a plasmid containing the GH fragment extending from positions 235 to 45 from the CAP site fused to the tk promoter. Lanes C and D represent the

30   extension product from cells transfected with a plasmid containing the tk promoter alone. No extension products were observed. The products shown in lanes A and C were from cells incubated in the absence of $T_3$ and those in lanes B and D were from cells treated with

35   $T_3$ at a concentration of $10^{-9}M$. Lane E shows a HindIII digest of pBR322, used for size calibration (in

nucleotides).

Figure VI-1 (A), (B) and (C) Methods.

Construction of CAT expression vectors
containing 5'-flanking sequences of the rat GH gene
5   from -1.7 kb to +8 bp of the CAP site has been
described[11]. Fusions containing the rat prolactin
enhancer were made by excising this fragment
(corresponding to rat prolactin sequence nucleotide
positions 1831-1530) from plasmid pPSS[11] and inserting
10  it in reverse orientation proximal to the 5' deletions
of the growth hormone element. Fusions containing the
HSV tk promoter were made by excising fragments of the
GH enhancer from the plasmid GPO (ref. 11) and
inserting them into the BamHI and SalI sites of a
15  pSV2CAT-based expression vectors proximal to the tk
promoter at positions -137 to +54. Alternatively, the
GH enhancer was placed proximal to the promoter at
positions -107 to +54 in a pUC8-based vector[20] by
insertion into the BamHI and SalI polylinker sites.
20  Site-directed mutagenesis of the GH enhancer element
was performed by inserting the fragment from 235-45 bp
from the CAP site into the BamHI and SalI sites of M13
mp18. A 21-base oligonucleotide was synthesized which
corresponded to antisense GH nucleotides -188 to -157,
25  in which nucleotides -177 to -166 were deleted and
replaced by an A nucleotide. This oligonucleotide was
used to delete bases -177 to -166 in the GH enhancer
using the procedure of Kunkel[21]. CAT activity was
determined by radioassay of methylated chloramphenicol
30  derivatives after thin layer chromatography[19]. Primer
extension was by the method of Elsholtz, et al.[12]

Figure VI-2 (A) and (B).

Binding of $T_3$ receptors to oligonucleotide
probes containing biotin-11-dUTP. A, Schematic
35  representation of two oligonucleotide probes used to
assay $T_3$ receptor binding to GH 5'-flanking sequences.

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Heavy lines, synthesized oligonucleotides with
complementary 3' ends.  Fine lines, bases incorporated
by filling the 5' overhangs using the large fragment of
Escherichia coli DNA polymerase.  Asterisks,
5    biotin-11-dUTP residues.  Restriction sites for BamHI
and BglII are also shown.  G209-146 and G186-158
contain rat GH enhancer sequences with the illustrated
5' and 3' boundaries.  B, Precipitation of $125_{I}-T_3$
labeled $T_3$ receptors from GC2 nuclear extracts by
10   various oligonucleotide probes containing
biotin-11-dUTP.  P-EGF, PB1-B, PB2-B, and PB4-B are
oligonucleotides of 68, 53, 55 and 58 base pairs
containing 10, 11, 10 and 10 biotin-11-dUTP's
respectively.  These oligonucleotides contain
15   5'-flanking sequences of the rat prolactin gene that
lack apparent homology to the rat GH sequence contained
in G209-186.  Precipitations were performed using 100
femtomole of each probe.  Background, representing $125_{I}$
activity associated with streptavidin agarose beads in
20   the absence of a biotinylated olignoucleotide probe,
was 1,400 c.p.m. in this experiment.  Results are
plotted as the mean and standard error of triplicate
points.  The experiment is representative of six
experiments examining the sequence specificity of
25   $125_{I}-T_3$ binding.

Figure VI-2 (A) and (B) Methods.

        Isolated nuclei were prepared from rat GC2
cells according to the technique of Dingham, et al.[22]
and salt extracted in 0.6 M KCl, 10 mM Hepes, pH 7.9,
30   0.5 mM dithiothreitol (DTT), 0.2 mM EGTA, 20 microM
$ZnCl_2$ for 30 min on ice.  The nuclear extract was
desalted by gel filtration in buffer A (50 mM KCl, 20
mM $K_3PO_1$, (pH 74.) 1 mM $MgCl_2$, 1 mM beta-mercaptoetaol
and 20% glycerol) and stored at -70°C.  Assay of
35   specific binding of $T_3$ to GC2 nuclear receptors was
performed as described by Samuels, et al.[23] except that

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T$_3$ binding reactions were performed in buffer A in the
presence of 200 micrograms ml$^{-1}$ poly(dI-dC).  To assay
DNA binding nuclear extracts were first incubated with
1 microM $^{125}$I-T$_3$(2,200 Ci mmol$^{-1}$) for 20 min at 22°C to
label the T$_3$ receptors to high specific activity.
Aliquots (40 microliters) of nuclear extract were then
incubated with biotinylated probes in the presence of
200 microliters poly(dI-dC) for 40 min at 22°C.
Protein-DNA complexes were precipitated by addition of
streptavidin conjugated to agarose beads (BRL).  The
agarose beads were pelleted, washed three times with
buffer A (1 ml) and assayed for $^{125}$I activity.
Figure VI-1.

        DNase I footprinting of the rat GH enhancer
element by GC2 nuclear extracts.  A 16-bp protected
region in the antisense strand is shown.  A second
footprint extending from position -110 to position -40
from the CAP site is also evident.  Lanes A-C,
Digestion of labeled GH enhancer after incubation with
GC2 nuclear extract, using 24, 12 and 4 microgram DNase
I respectively.  Lanes D-F, Digestion of labeled GH
enhancer with 24, 12 and 4 microgram of DNase I in the
absence of GC2 nuclear extract.  Lane G and M, Markers.
The displayed sequence corresponds to that of the sense
strand within the footprinted region.
Figure VI-3 Method.

        The antisense strand of the growth hormone
enhancer was labeled with $^{32}$P-dATP at its 5' end using
T4 polynucleotide kinase after BamHI digestion of pGPO
and treatment with calf intestinal phosphatase.  The
enhancer fragment was released from pGPO by XhoI
digestion and purified by agarose gel electrophoresis.
Labeled GH enhancer fragment (1 ng, 8 fmol) was
incubated with 25 microliters of GC2 nuclear extracts
containing 12 fmol of specific T$_3$ receptor-binding
activity.  The DNA binding reaction was carried out for

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30 min at 22°C in Buffer A.  DNase digestion was for 2
min at 22°C using the above concentrations of DNase I
in a final volume of 50 microliters.  The reactions
were stopped with 20 microliters 50 mM EDTA and 1% SDS.
5    Samples were extracted once with phenol-chloroform,
ethanol precipitated, and analyzed by electrophoresis
on standard 10% sequencing gels.
Figure VI-4.

        Binding to oligonucleotides containing 64 and
10   29 base pairs of 5' flanking GH sequence of rat
pituitary cell T3 and incubated with 100 fmol G209-146,
G186-158 or P-EGF and assayed for binding as described
in Fig. VI-2 (A) and (B).  Also, hc-erb-A in vitro
translation product (4 microliters) labeled with
15   35S-methionine was assayed for binding to these
oligonucleotides in the presence of 10 nM T3.  To
prepare the hc-erb-A in vitro translation product,
capped mRNA transcripts of an hc-erb-A complementary
DNA were used to program translation in a rabbit
20   reticulocyte lysate system[10]. Reticulocyte lysates
programmed with antisense hc-erb-A mRNA had no
measurable binding activity to either of the GH probes
(data not shown).  Results are plotted as mean and
standard error of triplicate points.  The experiments
25   shown is representative of three experiments comparing
the binding of GC2 nuclear T3 receptors to the two GH
probes and of four experiments examining the binding of
the hc-erb-A in vitro translation product.

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## EXPERIMENTAL SECTION VII
## IDENTIFICATION OF A NOVEL THYROID
## HORMONE RECEPTOR EXPRESSED IN THE
## MAMMALIAN CENTRAL NERVOUS SYSTEM

### VII. A. SUMMARY

A complementary DNA clone derived from rat
brain messenger RNA has been isolated on the basis of
homology to the human thyroid hormone receptor gene.
Expression of this complementary DNA produces a
high-affinity binding protein for thyroid hormones.
Sequence analysis and the mapping of this gene to a
distinct human genetic locus indicate the existence of
multiple human thyroid hormone receptors.  Messenger
RNA from this gene is expressed in a tissue-specific
fashion with highest levels in the central nervous
system.

### VII. B. INTRODUCTION

Thyroid hormones are involved in a complex
array of developmental and physiological responses in
many tissues of higher vertebrates (1).  Their numerous
and diverse effects include the regulation of important
metabolic enzymes, hormones, and receptors (2).  The
actions of thyroid hormones are mediated through a
nuclear receptor, which modulates the expression of
specific genes in target cells (3-5).  These properties
are similar to the interactions of steroid hormones
with their receptors and are consistent with the recent
observation of structural relatedness between steroid
and thyroid hormone receptors (see Experimental Section
III).

### VII. C. ISOLATION OF A SECOND THYROID RECEPTOR DNA

Despite the diversity of thyroid hormone
action, it is generally accepted that thyroid hormone
function occurs through a single high-affinity nuclear
receptor.  However, the recent characterization of the
thyroid hormone receptor as the cellular homolog of the

v-erb-A oncogene product (see Experimental Section III
and ref. 7), along with the previous identification of
multiple c-erb-A genes on human chromosomes 3 and 17
(see Experimental Section III and ref. 8), suggests the
5    existence of multiple thyroid hormone receptors.  To
examine the possibility that the mechanisms underlying
the multiple thyroid hormone responses may be derived
from the expression of structurally distinct thyroid
hormone receptors, we have isolated a complementary DNA
10   (cDNA) clone that encodes the product of one of these
related loci.

A putative neuronal form of the thyroid
hormone receptor was isolated by screening a cDNA
library prepared from rat brain messenger RNA (mRNA)
15   with a 1500-bp fragment of the human thyroid hormone
receptor cDNA.  (See Experimental Section III which has
been published as ref. 6.)  From ~$10^6$ phage, three
positive clones were isolated, and the complete
nucleotide sequence of the largest of these, rbeA12,
20   was determined (Fig. VII-1(B)).  The sequence is 2079
bp long and contains a long open reading frame of 1230
bp with a potential initiator methionine at nucleotide
position 325 and a terminator codon at position 1554.
This open reading frame is preceded by a 5'
25   untranslated region of at least 320 bp that contains
three short open reading frames upstream of the
putative initiator methionine and encodes a protein of
410 amino acid residues, with a calculated molecular
mass of 45 kD.
30   VII. D. COMPARISON OF THE SECOND THYROID
RECEPTOR WITH OTHER KNOWN THYROID RECEPTOR PROTEINS
Comparison of the deduced amino acid sequence
from rbeA12 with that of the human thyroid hormone
receptor (see Experimental Section III) reveals that
35   the two proteins have distinct amino termini (Fig.
VII-2).  The first 41 amino acids of the neuronal

protein and the first 90 amino acids of the human
thyroid hormone receptor show no significant homology,
whereas the carboxyl terminal 367 amino acids share 75%
nucleotide and 82% amino acid identities. The rat
5   protein is more related to the chicken thyroid hormone
receptor (7) both in predicted size and homology, and
shares 82% nucleotide and 89% amino acid identity. For
reference, the chicken thyroid hormone receptor is
designated alpha (cTR alpha) because of its homology to
10   previously isolated erb-A genes (8), and the human
thyroid hormone receptor is designated beta (hTR beta).
Because the rat neuronal form is more related to the
chicken receptor, it has been designated alpha (rTR
alpha).
15          By analogy to the steroid hormone receptors, a
cysteine-rich region in the thyroid hormone receptor is
predicted to be the DNA-binding domain (see
Experimental Sections III and II; also see ref. 10). In
this region, the rTR alpha protein has 97% amino acid
20   identity with the cTR alpha protein and 90% amino acid
identity with the hTR beta protein. The proteins are
also well conserved in the carboxyl terminal portion
that is presumed to be the hormone-binding domain,
again by analogy with the steroid receptors (see
25   Experimental Section II; also see ref. 11). This
region of rTR alpha shows 94% amino acid identity with
cTR alpha and 85% amino acid identity with hTR beta.

## VII. F. IDENTIFICATION OF THE NEW THYROID RECEPTOR

On the basis of the sequence data, it appears
30   that the cDNA we have isolated encodes a protein
different from the previously characterized human
thyroid hormone receptor (see Experimental Section
III). To demonstrate that the neuronal clone is a
distinct gene product, rbeA12 was used to identify
35   human homologs by Southern blot and chromosome
analyses. Human placenta DNA digested with various

restriction enzymes was separated on an agarose gel,
transferred to nitrocellulose, and hybridized with
either rat or human TR-specific probes derived from
overlapping regions of their respective genes (Fig.
5      VII-3 (A) and (B)).  Different hybridization patterns
were revealed for all of the restriction enzymes
tested, which indicates that the two cDNA's represent
distinct genes. The same probe from the rbeA12 was
hybridized to laser-sorted chromosomes prepared from
10     human lymphoid cells (Fig. VII-3(C)).  Hybridization
was observed only to chromosome 17, consistent with
previous mapping studies that localized c-erb-A genes
to human chromosome 17 (8).  This distinguishes rTR
alpha from hTR beta, which is found on human chromosome
15     3 (see Experimental Section III).

### VII. F. EXPRESSION STUDIES

Expression studies were performed to determine
whether the rTR alpha cDNA encodes a functional
receptor protein.  The product of the rTR alpha gene
20     was first characterized by in vitro transcription
followed by in vitro translation.  For in vitro
transcription, the EcoRI insert of rbeA12 was linked to
the bacteriophage SP6 promoter by subcloning into the
expression vector pGEM1.  A second construction,
25     rbeA12B, was created in an attempt to increase the
efficiency of translation.  The 5' untranslated region
up to nucleotide position 97 was deleted, which removed
two of the three short open reading frames in this
region.  Transcripts synthesized with SP6 polymerase
30     were translated in vitro with rabbit reticulocyte
lysates, and the [$^{35}$S]methionine-labeled products were
analyzed on an SDS-polyacrylamide gel (Fig. VII-4(A)).
Four proteins of approximately 52, 48, 35, and 33 kD
were observed only when the sense strand was
35     translated.  The same four bands were observed for
rbeA12 and rbeA12B.  These translation products were

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then used to test thyroid hormone binding.

### VII. G. HORMONE BINDING STUDIES

Thyroid hormone binding was measured with $[^{125}I]3,5,3'$-triiodo-L-thyronine ($^{125}I$-$T_3$). Only samples that contained the rTR alpha specific proteins exhibited $T_3$ binding. Hormone affinity was determined by Scatchard analysis, which gave a dissociation constant ($K_d$) of $2.9 \times 10^{-11}$M (Fig. VII-4(B)), similar to the $K_d$ observed for the hTR beta protein ($5 \times 10^{-11}$M) (4, 5) and an order of magnitude lower than that determined for the cTR alpha protein ($2.1 \times 10^{-10}$ to $3.3 \times 10^{-10}$M) (see Experimental Section III). The different $K_d$ values obtained may be due to differences in the assay systems used. In competition experiments, the rTR alpha proteins translated in vitro showed the same characteristic affinities for L-$T_3$ and L-thyroxine (L-$T_4$) as the hTR beta protein but revealed a different pattern for $3,5',3'$-triiodothyroacetic acid (TRIAC) (Fig. VII-4(C)). TRIAC competed better for $T_3$ binding with the hTR beta protein, whereas it competed about as well at $T_3$ for binding to the rTR alpha protein. As with the hTR beta and cTR alpha proteins, there was no competition for $T_3$ binding to the rTR alpha protein by excess aldosterone, estrogen, progesterone, testosterone, or vitamin $D_1$. Thus, it appears that we have isolated a thyroid hormone receptor with binding properties similar to but not identical to those of the thyroid hormone receptors previously described (see Experimental Section III and ref. 7).

### VII. H. TISSUE SPECIFICITY STUDIES

The tissue specificity of metabolic responses to thyroid hormone led us to consider that this thyroid hormone receptor might be expressed in a restricted set of tissues. Therefore, the pattern of expression of the rTR alpha gene was determined by Northern blot analysis (Fig. VII-5). Total RNA isolated from various

rat tissues was separated on a formaldehyde-agarose
gel, transferred to nitrocellulose, and hybridized to
the same fragment of rbeA12 used for the Southern blot
analysis and chromosome mapping.  A 2.6-kb RNA was
observed in all tissues tested except liver.  This
5       message is also present in pituitary and muscle and is
expressed in GC, rat-1, and PC12 cell lines.
Densitometric scanning indicated that the level of
expression of rTR alpha was 10- to 20-fold as high in
brain as in any other tissue tested.  Two additional
10      RNA's of approximately 5.0 and 6.0 kb are present in
about equal amounts in all tissues, although they are
much less abundant than the 2.6-kb message.  These
bands may represent precursors of the 2.6-kb message or
may be products of a related gene.

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### VII. L. DISCUSSION AND CONCLUSIONS

The isolation of a second mammalian thyroid
hormone receptor is surprising because previous
biochemical studies have not predicted the existence of
more than a single receptor for thyroid hormones.  In
20      retrospect, much of the clinical and physiological
studies can be interpreted as indicating the existence
of multiple receptors.  A form of functional
heterogeneity has been suggested by the identification
of patients with familial thyroid hormone resistance in
25      which peripheral response to thyroid hormones is lost
or diminished, while neuronal functions are maintained
(12, 13).  Furthermore, severe developmental effects
associated with low circulating thyroid hormone levels
(cretinism) have been classified into types severely
30      affecting the nervous system and those more
dramatically affecting peripheral functions (13, 14).

In addition to demonstrating the existence of
structurally distinct forms of the thyroid hormone
receptor, the form that we have characterized is
35      expressed at high levels in the rat central nervous

system. Preliminary studies utilizing in situ
hybridization have revealed high levels of expression
in the hippocampus, hypothalamus, cortex, and amygdala.
RNA hybridization studies indicate exceptionally high
5   levels in the cerebellum as well. Although it is known
that thyroid hormones play a critical role in early
brain development (14), this high level of expression
is unexpected because biochemical studies have shown
that brain has fewer thyroid hormone receptors than
10  many other tissues (5, 16), and the adult brain is not
responsive to thyroid hormone by traditional phosphate
dehydrogenase activity (17).

The second interesting result from the
expression studies is that this transcript is not
15  present in liver, which is the tissue from which
thyroid hormone receptors usually have been isolated.
This absence suggests the existence of yet another form
of the thyroid hormone receptor. This proposal would
be consistent with the data of Underwood, et al. (18),
20  which indicates the existence of pharmacologically
distinguishable thyroid hormone responses between liver
and heart. Furthermore, data from DNA hybridization
studies indicate the existence of multiple genetic loci
that hybridize with the cDNA clones for the mammalian
25  thyroid hormone receptor and suggest that there may be
as many as five different related loci (see
Experimental Section III and ref. 8). It seems likely
that some of these loci will encode additional
functional molecules, which leads us to propose the
30  existence of a family of thyroid hormone receptors that
coordinately regulate overlapping networks of genes to
control developmental and homeostatic function.

VII. J. DETAILED DESCRIPTION OF FIGURES
REFERRED TO IN EXPERIMENTAL SECTION VII
35  Figure VII-1 (A) and (B).

Restriction map and nucleotide and predicted

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amino acid sequence of thyroid hormone receptor cDNA
from rat brain.  (A) Schematic representation of
thyroid hormone receptor cDNA from rat brain; some
common restriction endonuclease cleavage sites are
5   indicated.  The hatched box indicates the predicted
coding region.  The 500-bp PvuII fragment
(corresponding to nucleotide positions 607 to 1113)
used for the hybridization studies is represented by
the solid bar below the restriction map.  (B) The
10  complete nucleotide sequence of rbeAl2 is shown with
the predicted amino acid sequence given above the long
open reading frame.  The three short open reading
frames in the 5' untranslated region are shown in bold
type with termination codons underlined.  Clone rbeAl2
15  was isolated by using the entire EcoRI insert of pheA4
(sigma) as a nick-translated probe to screen ⁻$10^6$ phage
from a rat brain cDNA library obtained from J. Arriza
(19).  Three positive clones were isolated, and the
complete nucleotide sequence of the largest of these,
20  rbeAl2, was determined on both strands by the chemical
cleavage method of Maxam and Gilbert (20).
Figure VII-2.

      Schematic comparison of the rat thyroid
hormone receptor (rTR alpha) protein with the human
25  thyroid hormone receptor (hTR beta) and chicken thyroid
hormone receptor (cTR alpha) proteins.  Numbers above
the boxes indicate amino acid residues; numbers inside
the boxes indicate the percent amino acid identity
within the enclosed region with the rTR alpha protein.
30  DNA designates the putative DNA-binding domain
predicted by analogy with the human glucocorticoid
receptor (amino acids 421 to 486 of the human
glucocorticoid receptor), while $T_3/T_4$ designates the
putative hormone-binding domain.
35  Figure VII-3 (A), (B) and (C).

      Southern blot analysis and human chromosomal

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localization of the rTR alpha gene.  Human placenta DNA
was digested with various restriction enzymes,
separated on a 0.8% agarose gel, transferred to
nitrocellulose, and hybridized to either a 500-bp PvuII

5   fragment from rbeA12 (A) or a 450-bp SatI fragment from
hTR beta (see Experimental Section III) that
encompasses the DNA-binding region (B).  Both blots
were hybridized in 50% formamide, 5 x SSPE (0.15M NaCl,
0.01M NaH$_2$PO$_4$, 0.001M EDTA), 1 x Denhardt's solution,

10  0.1% SDS, and salmon sperm DNA (100 micrograms/ml) at
42°C, and washed in 2 x SSC (standard saline citrate)
and 0.1% SDS at 68°C.  Sizes of lambda HindIII markers
in kilobase pairs are indicated (C).  Chromosome
mapping of the rTR alpha gene.  Human lymphocyte

15  chromosomes were separated by laser cytofluorometry
(21) and hybridized under the same conditions as above
with the 500-bp PvuII fragment of rbeA12.
Figure VII-4 (A), (B) and (C).

        In vitro translation and thyroid hormone

20  binding of rTR alpha (A) rTR alpha was transcribed in
vitro and translated in a rabbit reticulocyte lysate.
The [$^{35}$S]methionine-labeled products were separated on
a 7.5% SDS-polyacrylamide gel and visualized by
fluorography.  Lane 1, no added RNA; lane 2, rbeA12,

25  which contains the entire 5' untranslated region; lane
3, rbeA12B, which contains only 97 bp of 5'
untranslated sequence.  Sizes of protein markers:
bovine serum albumin, 66.2 kD; ovalbumin, 45 kD;
carbonic anhydrase, 31 kD. (B) Scatchard analysis of

30  $^{125}$I-T$_3$ binding to in vitro translated rTR alpha
Lysates containing in vitro translated rbeA12B
transcripts were assayed for specific thyroid
hormone-binding activity by measuring the amount of
hormone bound at different concentrations of

35  $^{125}$I-T$_3$-K$_d$= 2.9 x 10$^{-11}$M. (C)  Competition of thyroid
hormone analogs for $^{125}$I-T$_3$ binding to in vitro

translated rTR alpha.  Samples from rbeA12B programmed
lysates were mixed with increasing concentrations of
unlabeled thyroid hormone or analogs to compete with
labeled hormone.  Specifically bound $^{125}I-T_3$ is plotted
5  versus conce.tration of competitor compound.  The same
competition pattern was obser d in four separate
experiments.  In _vitro_ transcription and translation
and hormone binding were performed as described (22,
23).  Open circles represent TRIAC; solid circles
10  represent L-$T_3$; solid triangles represent L-$T_4$.
Figure VII-5.

        Tissue distribution of rTR alpha mRNA.  Total
RNA was isolated from various rat tissues with
guanidine thiocyanate (24), separated on a 1%
15  agarose-formaldehyde gel, transferred to
nitrocellulose, and hybridized with a nick-translated
500-bp PvuII fragment from rbeA12.  The tissue type and
the amount of total RNA loaded are indicated above each
lane.  A cDNA of CHO-B, a Chinese hamster ovary cell
20  mRNA expressed at equivalent levels in all tissues
examined (25), was used as an internal standard.
Positions of 28S and 18S ribosomal RNA are indicated.

### VII. K. REFERENCES REFERRED TO IN
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means of oligo(dT)-selected RNA from whole rat
brain. Moloney murine leukemia virus (M-MuLV)
reverse transcriptase (RT) was used for first
strand synthesis; second strand synthesis was
with the Klenow fragment of DNA polymerase I
followed by M-MuLV RT-cDNAs were treated with
SI nuclease, methylated, size-fractionated on
Sepharose 4B, ligated to lambda gtII arms, and
packaged. The library consists of $25 \times 10^6$
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insert of rbeAl2 was cloned into pGEM1
(Promega Biotec). A second construction
deleting 227 bp of 5' untranslated sequence,

rbeA12B, was made by inserting the $T_4$DNA polymerase-filled BgI-SmaI fragment of rbeA12 (nucleotide position 227 to the polylinker) into the SmaI site of pGEM3. For thyroid

5      hormone binding, transcriptions were performed with SP6 polymerase and 5 to 10 micrograms of rbeA12B linearized with SstI. Transcripts were purified by P60 chromatography and translated in 150 to 200 microliters of rabbit

10      reticulolyte lysate (Promega Biotec) in conditions suggested by the manufacturer. Thyroid hormone binding for both the Scatchard and competition analyses were determined in the same manner, except that unlabeled protein

15      was used for the Scatchard analysis. [$^{125}$I]3,3',5:Triodothyronine (New England Nuclear, 2200 Ci/mmol, 0.3nM final concentration) was mixed with rTR alpha polypeptides synthesized in vitro (5 to 8

20      microliters of the 200 microliters of lysate per binding reaction) in $T_3$ binding buffer at 0°C for 2 hours in a final volume of 250 microliters. Specific hormone binding was determined by adding a 1000-fold excess of

25      unlabeled hormone and assayed by counting radioactivity eluting in the excluded volume from a Sephadex G-25 fine (Pharmacia) 0.9-by 4.0-cm. column.

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35

## SPECIFICATION SUMMARY

From the foregoing description, one of
ordinary skill in the art can understand that the
present invention provides substantially pure DNA's
comprised of sequences which encode proteins having the
hormone-binding and/or transcription-activating
characteristics of a glucocorticoid receptor, a
mineralocorticoid receptor, or a thyroid hormone
receptor.  The invention also provides various plasmids
containing receptor sequences which exemplify the DNA's
of the invention.  Exemplary plasmids of the invention
have been deposited with the American Type culture
Collection for patent purposes.

The invention is also comprised of receptor
proteins, including modified functional forms thereof,
expressed from the DNA's (or mRNA's) of the invention.

In addition to novel receptor DNA, RNA and
protein compositions, the present invention involves a
bioassay for determining the functionality of a
receptor protein.  It also involves two new methods for
producing desired proteins in genetically engineered
cells.  The first is a method for inducing
transcription of a gene whose transcription is
activated by hormones complexed the receptors.  The
second is a method for engineering a cell and then
increasing and controlling production of a protein
encoded by a gene whose transcription is activated by
hormones complexed with receptor proteins.

The DNA's of the invention can be used to make
the hormone receptor proteins, and functional modified
forms thereof, in quantities that were not previously
possible.  With the quantities of receptor available as
a result of the present invention, detailed structural
analyses of the proteins can now be made by using X-ray
diffraction methods to analyze receptor crystals.  In
addition, adequate supplies of the receptor proteins

mean that they can now be used to screen compounds for
receptor-agonists or receptor-antagonist activity.
Availability of the receptor proteins also means that
they can be used in diagnostic assays.

5      Without departing from the spirit and scope of
this invention, one or ordinary skill can make various
changes and modifications to the invention to adapt it
to various usages and conditions. As such, these
changes and modifications are properly, equitable, and

10   intended to be, within the full range of equivalence of
the following claims.

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International Application No: PCT/    /

## MICROORGANISMS

Optional Sheet in connection with the microorganism referred to on page _____, line _____ of the description [¹]

**A. IDENTIFICATION OF DEPOSIT** [¹]

Further deposits are identified on an additional sheet ☐ [²]

Escherichia coli HB101
pRShGR

Name of depository institution [⁴]

AMERICAN TYPE CULTURE COLLECTION

Address of depository institution (including postal code and country) [⁴]

12301 Parklawn Drive
Rockville, Maryland 20852 U.S.A.

| Date of deposit [⁴] | Accession Number [⁴] |
|---|---|
| September 9, 1986 | 67200 |

**B. ADDITIONAL INDICATIONS** [¹] (leave blank if not applicable). This information is continued on a separate attached sheet ☐

In respect of those designations in which a European
patent is sought, a sample of the deposited microorganism
will be made available until the publication of the men-
tion of the grant of the European patent or until the
date on which the application has been refused or with-
drawn or is deemed to be withdrawn, only by the issue of
such a sample to an expert nominated by the person
requesting the sample. (Rule 28(4) EPC)

**C. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE** [¹] (if the indications are not for all designated States)

EUROPEAN PATENT OFFICE

**D. SEPARATE FURNISHING OF INDICATIONS** [¹] (leave blank if not applicable)

The indications listed below will be submitted to the International Bureau later [¹] (Specify the general nature of the indications e.g.,
"Accession Number of Deposit")

E. ☐ This sheet was received with the international application when filed (to be checked by the receiving Office)

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☐ The date of receipt (from the applicant) by the International Bureau was

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(Authorized Officer)

(January 1985)

-174-                                                           ANNEX M3

## MICROORGANISMS

Optional Sheet in connection with the microorganism referred to on page_____ line_____ of the description

**A. IDENTIFICATION OF DEPOSIT**

Further deposits are identified on an additional sheet ☐

Escherichia coli HB101
pRShMR

Name of depositary institution

AMERICAN TYPE CULTURE COLLECTION

Address of depositary institution (including postal code and country)

12301 Parklawn Drive
Rockville, Maryland 20852 U.S.A.

| Date of deposit | Accession Number |
|---|---|
| September 9, 1986 | 67201 |

**B. ADDITIONAL INDICATIONS** (leave blank if not applicable. This information is continued on a separate attached sheet ☐

In respect of those designations in which a European
patent is sought, a sample of the deposited microorganism
will be made available until the publication of the men-
tion of the grant of the European patent or until the
date on which the application has been refused or with-
drawn or is deemed to be withdrawn, only by the issue of
such a sample to an expert nominated by the person
requesting the sample. (Rule 28(4) EPC)

**C. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE** (if the indications are not for all designated States)

EUROPEAN PATENT OFFICE

**D. SEPARATE FURNISHING OF INDICATIONS** (leave blank if not applicable)

The indications listed below will be submitted to the International Bureau later (Specify the general nature of the indications e.g.,
"Accession Number of Deposit")

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(Authorized Officer)

(January 1985)

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ANNEX M3

International Application No: PCT/ /

## MICROORGANISMS

Optional Sheet in connection with the microorganism referred to on page _____, line _____ of the description

**A. IDENTIFICATION OF DEPOSIT**

Further deposits are identified on an additional sheet ☐

Escherichia coli HB101, pcA101

Name of depositary institution

AMERICAN TYPE CULTURE COLLECTION

Address of depositary institution (including postal code and country)

12301 Parklawn Drive
Rockville, Maryland 20852 U.S.A.

| Date of deposit | Accession Number |
|---|---|
| October 22, 1986 | 67244 |

**B. ADDITIONAL INDICATIONS** (leave blank if not applicable. This information is continued on a separate attached sheet ☐)

In respect of those designations in which a European
patent is sought, a sample of the deposited microorganism
will be made available until the publication of the men-
tion of the grant of the European patent or until the
date on which the application has been refused or with-
drawn or is deemed to be withdrawn, only by the issue of
such a sample to an expert nominated by the person
requesting the sample. (Rule 28(4) EPC)

**C. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE** (if the indications are not for all designated States)

EUROPEAN PATENT OFFICE

**D. SEPARATE FURNISHING OF INDICATIONS** (leave blank if not applicable)

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ANNEX M3

-176-

International Application No: PC.,; /

## MICROORGANISMS

Optional Sheet in connection with the microorganism referred to on page _____ line _____ of the description

**A. IDENTIFICATION OF DEPOSIT**

Further deposits are identified on an additional sheet ☐

Escherichia coli
JB101, rbcAl2

Name of depositary institution

AMERICAN TYPE CULTURE COLLECTION

Address of depositary institution (including postal code and country)

12301 Parklawn Drive
Rockville, Maryland 20852 U.S.A.

| Date of deposit | Accession Number |
|---|---|
| December 18, 1986 | 67281 |

**B. ADDITIONAL INDICATIONS** (leave blank if not applicable. This information is continued on a separate attached sheet ☐)

In respect of those designations in which a European patent is sought, a sample of the deposited microorganism will be made available until the publication of the mention of the grant of the European patent or until the date on which the application has been refused or withdrawn or is deemed to be withdrawn, only by the issue of such a sample to an expert nominated by the person requesting the sample. (Rule 28(4) EPC)

**C. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE** (if the indications are not for all designated States)

EUROPEAN PATENT OFFICE

**D. SEPARATE FURNISHING OF INDICATIONS** (leave blank if not applicable)

The indications listed below will be submitted to the International Bureau later (Specify the general nature of the indications e.g. "Accession Number of Deposit")

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(January 1985)

Form PCT/RO/134 (January 1981)

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International Application No: PCT/                    /

## MICROORGANISMS

Optional Sheet in connection with the microorganism referred to on page_____ line_____ of the description !

**A. IDENTIFICATION OF DEPOSIT** *

Further deposits are identified on an additional sheet ☐ *

<u>Escherichia coli</u>
HB101, GMCAT

Name of depositary institution *

AMERICAN TYPE CULTURE COLLECTION

Address of depositary institution (including postal code and country) *

12301 Parklawn Drive
Rockville, Maryland 20852 U.S.A.

| Date of deposit * | Accession Number * |
|---|---|
| December 18, 1986 | 67282 |

**B. ADDITIONAL INDICATIONS** * (leave blank if not applicable). This information is continued on a separate attached sheet ☐

In respect of those designations in which a European
patent is sought, a sample of the deposited microorganism
will be made available until the publication of the men-
tion of the grant of the European patent or until the
date on which the application has been refused or with-
drawn or is deemed to be withdrawn, only by the issue of
such a sample to an expert nominated by the person
requesting the sample. (Rule 28(4) EPC)

**C. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE** * (if the indications are not for all designated States)

EUROPEAN PATENT OFFICE

**D. SEPARATE FURNISHING OF INDICATIONS** * (leave blank if not applicable).

The indications listed below will be submitted to the International Bureau later * (Specify the general nature of the indications e.g.,
"Accession Number of Deposit")

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(January 1985)

ANNEX H3

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International Application No: PCT/                    /

## MICROORGANISMS

Optional Sheet in connection with the microorganism referred to on page _____ line _____ of the description

**A. IDENTIFICATION OF DEPOSIT**

Further deposits are identified on an additional sheet ☐

Escherichia coli
DH5, pE4

Name of depositary institution

AMERICAN TYPE CULTURE COLLECTION

Address of depositary institution (including postal code and country)

12301 Parklawn Drive
Rockville, Maryland 20852 U.S.A.

| Date of deposit | Accession Number |
|---|---|
| January 30, 1987 | 67309 |

**B. ADDITIONAL INDICATIONS** (leave blank if not applicable). This information is continued on a separate attached sheet ☐

In respect of those designations in which a European
patent is sought, a sample of the deposited microorganism
will be made available until the publication of the men-
tion of the grant of the European patent or until the
date on which the application has been refused or with-
drawn or is deemed to be withdrawn, only by the issue of
such a sample to an expert nominated by the person
requesting the sample. (Rule 28(4) EPC)

**C. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE** (if the indications are not for all designated States)

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**D. SEPARATE FURNISHING OF INDICATIONS** (leave blank if not applicable)

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(January 1985)

-179-                              •                    ANNEX H3

International Application No: PCT/                              /

## MICROORGANISMS

Optional Sheet in connection with the microorganism referred to on page_____, line_____, of the description [1]

**A. IDENTIFICATION OF DEPOSIT** [1]

Further deposits are identified on an additional sheet ☐ [1]

Escherichia coli
DH5, pHKA

Name of depositary institution [1]

AMERICAN TYPE CULTURE COLLECTION

Address of depositary institution (including postal code and country) [1]

12301 Parklawn Drive
Rockville, Maryland 20852 U.S.A.

| Date of deposit [1] | Accession Number [1] |
|---|---|
| January 30, 1987 | 67310 |

**B. ADDITIONAL INDICATIONS** [1] (leave blank if not applicable). This information is continued on a separate attached sheet ☐

In respect of those designations in which a European
patent is sought, a sample of the deposited microorganism
will be made available until the publication of the men-
tion of the grant of the European patent or until the
date on which the application has been refused or with-
drawn or is deemed to be withdrawn, only by the issue of
such a sample to an expert nominated by the person
requesting the sample. (Rule 28(4) EPC)

**C. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE** [1] (if the indications are not for all designated States)

EUROPEAN PATENT OFFICE

**D. SEPARATE FURNISHING OF INDICATIONS** [1] (leave blank if not applicable)

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(January 1985)

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International Application No: PCT/                        /

## MICROORGANISMS

Optional Sheet in connection with the microorganism referred to on page _____, line _____ of the description *

**A. IDENTIFICATION OF DEPOSIT** *

Further deposits are identified on an additional sheet ☐ *     Plasmid DNA phERBA8.7

Name of depositary institution *

AMERICAN TYPE CULTURE COLLECTION

Address of depositary institution (including postal code and country) *

12301 Parklawn Drive
Rockville, Maryland 20852 U.S.A.

| Date of deposit * | Accession Number * |
|---|---|
| September 29, 1987 | 40374 |

**B. ADDITIONAL INDICATIONS** * (leave blank if not applicable. This information is continued on a separate attached sheet ☐

In respect of those designations in which a European
patent is sought, a sample of the deposited microorganism
will be made available until the publication of the men-
tion of the grant of the European patent or until the
date on which the application has been refused or with-
drawn or is deemed to be withdrawn, only by the issue of
such a sample to an expert nominated by the person
requesting the sample. (Rule 28(4) EPC)

**C. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE** * (if the indications are not for all designated States)

EUROPEAN PATENT OFFICE

**D. SEPARATE FURNISHING OF INDICATIONS** * (leave blank if not applicable)

The indications listed below will be submitted to the International Bureau later * (Specify the general nature of the indications e.g.,
" Accession Number of Deposit ")

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(Authorized Officer)

(January 1981)

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ANNEX H3

## MICROORGANISMS

Optional Sheet in connection with the microorganism referred to on page_____, line_____ of the description.

**A. IDENTIFICATION OF DEPOSIT**

Further deposits are identified on an additional sheet ☐ . Plasmid DNA, phFA8

Name of depositary institution

AMERICAN TYPE CULTURE COLLECTION

Address of depositary institution (including postal code and country)

12301 Parklawn Drive
Rockville, Maryland 20852 U.S.A.

| Date of deposit | Accession Number |
|---|---|
| September 29, 1987 | 40372 |

**B. ADDITIONAL INDICATIONS** (leave blank if not applicable). This information is continued on a separate attached sheet ☐

In respect of those designations in which a European
patent is sought, a sample of the deposited microorganism
will be made available until the publication of the men-
tion of the grant of the European patent or until the
date on which the application has been refused or with-
drawn or is deemed to be withdrawn, only by the issue of
such a sample to an expert nominated by the person
requesting the sample. (Rule 28(4) EPC)

**C. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE** (if the indications are not for all designated States)

EUROPEAN PATENT OFFICE

**D. SEPARATE FURNISHING OF INDICATIONS** (leave blank if not applicable)

The indications listed below will be submitted to the International Bureau later (Specify the general nature of the indications e.g.,
"Accession Number of Deposit")

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(Authorized Officer)

☐ The date of receipt (from the applicant) by the International Bureau is

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(Authorized Officer)

Form PCT/RO/134 (January 1981)

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International Application No: PCT/          /

## MICROORGANISMS

Optional Sheet in connection with the microorganism referred to on page _____ line _____ of the description

**A. IDENTIFICATION OF DEPOSIT**

Further deposits are identified on an additional sheet ☐    Plasmid DNA, phH3

Name of depositary institution

AMERICAN TYPE CULTURE COLLECTION

Address of depositary institution (including postal code and country)

12301 Parklawn Drive
Rockville, Maryland 20852 U.S.A.

| Date of deposit | Accession Number |
|---|---|
| September 29, 1987 | 40373 |

**B. ADDITIONAL INDICATIONS** (leave blank if not applicable). This information is continued on a separate attached sheet ☐

In respect of those designations in which a European
patent is sought, a sample of the deposited microorganism
will be made available until the publication of the men-
tion of the grant of the European patent or until the
date on which the application has been refused or with-
drawn or is deemed to be withdrawn, only by the issue of
such a sample to an expert nominated by the person
requesting the sample. (Rule 28(4) EPC)

**C. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE** (if the indications are not for all designated States)

EUROPEAN PATENT OFFICE

**D. SEPARATE FURNISHING OF INDICATIONS** (leave blank if not applicable)

The indications listed below will be submitted to the International Bureau later (Specify the general nature of the indications e.g.,
"Accession Number of Deposit")

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(Authorized Officer)

Form PCT/RO/134 (January 1981)

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WHAT IS CLAIMED IS:

1.    A substantially pure double-stranded DNA
wherein the sense strand comprises a segment having a
sequence of triplets coding for the primary sequence of
a protein which has hormone-binding and/or
transcription-activating properties characteristic of a
hormone receptor selected from the group consisting of
a glucocorticoid receptor, a mineralocorticoid receptor
and a thyroid hormone receptor, said double-stranded
DNA segment being capable of being expressed into said
protein.

2.    A substantially pure DNA according to
Claim 1 wherein the sequence of triplets in said
segment codes for the primary sequence of a protein
which has hormone-binding and/or
transcription-activating properties characteristic of a
glucocorticoid receptor.

3.    A substantially pure DNA according to
Claim 1 wherein the sequence of triplets in the segment
codes for the primary sequence of a protein which has
hormone-binding and/or transcription-activating
properties characteristic of a mineralocorticoid
receptor.

4.    A substantially pure DNA according to
Claim 1 wherein the sequence of triplets in the segment
codes for the primary sequence of a protein which has
hormone-binding and/or transcription-activating
properties characteristic of a thyroid hormone
receptor.

5.    A substantially pure DNA according to
Claim 2 wherein the sequence of triplets in the segment
codes for the primary sequence of a protein which has
hormone-binding and/or transcription-activating
properties characteristic of an avian or mammalian
glucocorticoid receptor.

6. A substantially pure DNA according to
Claim 5 wherein the sequence of triplets in the segment
codes for the primary sequence of a protein which has
hormone-binding and/or transcription-activating
properties characteristic of the human glucocorticoid
receptor.

7. A substantially pure DNA according to
Claim 3 wherein the sequence of triplets in the segment
codes for the primary sequence of a protein which has
hormone-binding and/or transcription-activating
properties characteristic of a mammalian or an avian
mineralocorticoid receptor.

8. A substantially pure DNA according to
Claim 3 wherein the sequence of triplets in the segment
codes for the primary sequence of a protein which has
hormone-binding and/or transcription-activating
properties characteristic of a human mineralocorticoid
receptor.

9. A substantially pure DNA according to
Claim 4 wherein the sequence of triplets in the segment
codes for the primary sequence of a protein which has
hormone-binding and/or transcription-activating
properties characteristic of a mammalian or an avian
thyroid hormone receptor.

10. A substantially pure DNA according to
Claim 4 wherein the sequence of triplets in the segment
codes for the primary sequence of a protein which has
hormone-binding and/or transcription-activating
properties characteristic of the human thyroid
receptor.

11. A substantially pure DNA according to
Claim 10 wherein said thyroid hormone receptor is
selected from the group consisting of human thyroid
receptor alpha and human thyroid receptor beta.

12. A plasmid selected from the group
consisting of: pRShGR alpha (ATCC # 67200), pRShMR

(ATCC # 67201), peA101 (ATCC # 67244), rbe A12 (ATCC # 67281), GMCAT (ATCC #67282) PE4 (ATCC # 67309), pHRA (ATCC # 67310), phERBA 8.7 (ATCC # 40374), phFA 8 (ATCC # 40372, and phH 3 (ATCC # 40373).

13.    A DNA selected from the group consisting of pRShGR-alpha (ATCC # 67200), the pRShGR-alpha modifications 19, 137, 1102, 1120, 1204, 1214, 1262, 1289, 1305, 1346, 1384, 1403, 1408, 1422, 1428, 1440, 1488, 1490, 1515, 1532, 1550, and 1684.

14.    A DNA segment which has (a) one triplet corresponding to a translation stop codon, which is in frame with a triplet corresponding to a translation start codon and downstream of said start codon triplet, and (b) as longest open reading frame, a substantially pure segment with a sequence of triplets coding for the primary sequence of a protein selected from the group consisting of: human glucocorticoid receptor and the modifications thereof expressed from the pRShGR-alpha, said modifications being known as modifications 19, 137, 1102, 1120, 1204, 1214, 1262, 1289, 1305, 1346, 1384, 1403, 1408, 1422, 1428, 1440, 1488, 1490, 1515, 1532, 1550, and 1684; human mineralocorticoid receptor; human thyroid receptor, and human steroid receptor known as hERR1 and hERR2.

15.    A substantially pure DNA according to Claim 14 wherein said human thyroid receptor is an alpha or beta receptor selected from the group con- sisting of DNA known as hERBA 8.7, hFA 8, and peA101.

16.    A substantially pure DNA sequence selected from the group of DNA sequences consisting of the DNA sequences shown in Figures I-2(1) and I-2(2) (hGR), III-1(B)-1 and III-1(B)-2 (hTR beta), III-7 (hTR alpha), IV-2(B)-1 and IV-2(B)-2 (hMR), V-1(B)-1 and V-1(B)-2 (hERR1), and V-2(B)-1 and V-2(B)-2 (hERR2).

17.    A substantially pure primary protein sequence comprised of an amino acid sequence selected

from the group consisting of the amino acid sequences
shown in Figures III-1(B) (hTR beta), III-7 (hTR
alpha), IV-2'B) (hMR), V-1(B) (hERR1), and V-2(B)
(hERR2).

18.  A substantially pure DNA encoding human
mineralocorticoid receptor.

19.  A substantially pure DNA encoding human
thyroid receptor wherein said receptor is selected from
the group consisting of human thyroid receptor alpha
and human thyroid receptor beta.

20.  A substantially pure DNA encoding
estrogen-related receptors wherein said receptors are
selected from the group consisting of hERR1 and hERR2.

21.  A DNA having substantial sequence homolo-
gy with any of the DNA's claimed in any of Claims 1-20.

22.  A mutant of any of the DNA's claimed in
any of Claims 1-20.

23.  An mRNA transcribed from any of the
substantially pure DNA sequences claimed in any of
Claims of 1-20.

24.  A substantially pure protein encoded by
any of the substantially pure DNA sequences claimed in
any of Claims 1-20.

25.  A substantially pure receptor protein
selected from the group consisting of mammalian and
avian thyroid receptor, mammalian and avian
mineralocorticoid receptor, and mammalian and avian
steroid-related receptors hERR1 and hERR2.

26.  A substantially pure thyroid receptor
according to Claim 25 wherein said thyroid receptor is
selected from the group consisting of thyroid receptor
alpha and thyroid receptor beta.

27.  Cells transformed by any of the
substantially pure DNA's claimed in any of Claims 1-20.

28.  A bioassay for determining the
functionality of a hormone receptor (ˉ) or an

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engineered functional modified form (r) thereof wherein
said functional modified form (r) has the
transcription-activating properties of receptor (R),
and wherein (R) and (r) are encoded by an expressible
5    DNA sequence, said bioassay comprising

(a) cotransfecting into receptor-negative
cells a first plasmid comprised essentially of an
operative hormone responsive promoter/enhancer element
functionally linked to an operative reporter gene, and
10   a second receptor expression plasmid comprised
essentially of an operative DNA sequence coding for
hormone receptor protein (R) or a functional modified
form (r) thereof,

(b) culturing said cotransfected cells from
15   step (a) in the presence and absence of a hormone, or
analog thereof, able to activate said hormone
responsive promoter/enhancer element of said first
plasmid,

(c) monitoring in said cell induction of the
20   product of said reporter gene DNA sequence, and

(d) measuring in said cells the expression of
and the steroid-binding capacity of the hormone
receptor protein (R) or functional modified form (r)
thereof encoded by said DNA sequence of said second
25   plasmid.

29.  A bioassay according to Claim 28 wherein
said receptor-negative cells are selected from the
group consisting of CV-1 and COS cells.

30.  A bioassay according to Claim 28 wherein
30   said first and second plasmids also contain the origin
of replication of SV-40.

31.  A bioassay according to Claim 28 wherein
said first and second plasmids also contain a
selectable marker.
35   32.  A bioassay according to Claim 28 wherein
said operative hormone responsive promoter/enhancer

element is selected from the group consisting of mouse mammary tumor virus long terminal repeat (MTV LTV) and and mammalian growth hormone promoter, and said reporter gene is CAT.

33.    A bioassay according to Claim 28 wherein hormone receptor protein (R) is a receptor selected from the group consisting of glucocorticoid receptor (GR), mineralocorticoid receptor (MR) and thyroid receptor (TR).

34.    In a cell (C) which contains gene (G), a gene whose transcription is substantially activated by hormone (H), or an analog (aH) thereof, when (H) or (aH) are complexed with receptor (R) or a functional modified form (r) thereof, and when said [(H) or (aH)/(R) or (r)] complex is bound to a transcriptional control element on the chromatin where gene (G) is located, a method for inducing expression of gene (G) and consequently production of the protein (P) coded for by gene (G), said method comprising: (1), expressing in cell (C) a DNA encoding receptor (R) or a modified functional form (r) thereof which has the transcription-activating properties of receptor (R), and (2), increasing the concentration of hormone (H) or analog (aH) in cell (C) to a level sufficient to induce expression of gene (G), and consequently production of protein (P).

35.    A method according to Claim 34 wherein said increase in said concentration of said hormone (H) or analog (aH) is controlled so as to control transcription of gene (G), and thus production of protein (P).

36.    A method according to any of Claims 34 and 35 wherein said cell (C) is a eukaryotic cell.

37.    A method according to any of Claims 34 and 35 wherein said eukaryotic cell (C) is a mammalian cell.

38.   A method according to any of Claims 34
and 35 wherein said eukaryotic cell (C) is a human
cell.

39.   A method according to any of Claims 34
and 35 wherein hormone (H) is a hormone selected from
the group consisting glucocorticoid hormones,
mineralocorticoid hormones and thyroid hormones, and
analogs thereof.

40.   A method according to any of Claims 34
and 35 wherein receptor (R) is a receptor selected from
the group consisting of glucocorticoid receptor,
mineralocorticoid receptor, and thyroid receptor.

41.   A method for engineering a cell and
producing a protein (P) that is encoded by a gene (G),
a gene whose transcription is substantially activated
by hormone (H), or an analog (aH) thereof, when (H) or
(aH) are complexed with receptor (R) or a functional
modified form (r) thereof, and when said [(H) or
(aH)/(R) or (r)] complex is bound to a transcriptional
control element on the chromatin where gene (G) is
located, said method comprising: (1), placing gene (G)
in cell (C) so that gene (G) is under the control of a
transcriptional control element that can be activated
by a [(H) or (aH)/(R) or (r)] complex when bound to
said transcriptional control element; (2), transforming
cell (C) with a DNA coding for receptor (R) or a
functional modified form (r) thereof which has the
transcription-activating properties of receptor (R),
and; (3), increasing the concentration of hormone (H)
or analog (aH) in cell (C) to a level sufficient to
induce and control expression of gene (G), and
consequently production of protein (P).

42.   A method according to Claim 41 wherein
said cell (C) is a eukaryotic cell.

43.   A method according to Claim 42 wherein
said eukaryotic cell (C) is a mammalian cell.

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44. A method according to any of Claim 43 wherein said eukaryotic cell (C) is a human cell.

45. A method according to Claim 41 wherein hormone (H) is a hormone selected from the group consisting glucocorticoid hormones, mineralocorticoid hormones and thyroid hormones, and analogs thereof.

46. A method according to Claim 41 wherein receptor (R) is a receptor selected from the group consisting of glucocorticoid receptor, mineralocorticoid receptor, and thyroid receptor.

FIG. I-1(A)

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FIG. I-1(B)

## FIG. 1-2(1)

## FIG. I-2(2)

FIG. I-3(A)

AIG

Common

TGA

Bal I
Bal I
Bst I
Sst I
Eco RI
Nco I
Nco I
Xba I
Hind III

2500    3000    3500

FIG. I-3(B)

α

```
                           710                                   740
ValValGluAsnLeuLeuAsnTyrCysPheGlnThrPheLeuAspLysThrMetSerIle
GTGGTTGAAAATCTCCTAACTATGCTTCTTCAAACATTTTGGATAAGACCATGAGTATT      2373

                750                                   760
GluPheProGluMetLeuAlaGluIleIleThrAsnGlnIleProLysTyrSerAsnGly
GAATTCCCCGAGATGTTAGCTGAAATCATCACCAATCAGATACCAAAATATTCAAATGGA    2433

                770
AsnIleLysLeuLeuPheHisGlnLysSTOP
AATATCAAAAAACTCTGTTTCATCAAAAGTGA......
```

```
   720
--GlnLeuThrLysLeuLeuAspSerMetHisGlu
--GGTCTGACAAAACCTTGGATTCTATGCATGAA
```

β

```
                           730                                   740
AsnValMetTrpLeuLysProGluSerThrSerHisThrLeuIleSTOP
AATGTTATGTGGTTAAAACCAGAAAGCACATCTCACACATTAATCTGATTTTCATCCCAA    2373
```

```
CAATCTTGGGCTCAAAAAATGCAACTCAATGAGAAAAGAAGATTATGTGCACTTCGTT       2433
AGTCATCTTAATTATGTTAATTGTGGATTTATTTGAAGCAGAGTTTATTTCGTGTGGTCTC    2633
CCCAAACTGTGAAGTTTGTAGTAACTTCAGTGAGMGTGGTGGTTACTCACACAACACTTT    2833
ATACAAGCAGAACTGAGGCACTTGAAGACTATAACACTTTGGGTATATATCCAAATGGAAACCT  2933
AACTAACATGATTTGTGTCTATGAGTGCTGGATAATTAGCATGGGATGAGTCTGGATC      3033
TAGGGGAGCAGCTTCCAGTGCTTCTCAACTTCCAGACCATGTGAGCCTTCAGTGAGTT      3233
TACATTCATCTTCAAACTTCAAACTTCCAGAACCATGGTAGCCTTCAGTTCCCTTTAAAA    3333
GTGTGTGTGTCGGTTTAGTGTCAGAAGGGGAATTAAAGTGTAAGGCAGACCTTTTCAGG      3433
CAAGCAACTGGTCGTCACCCAGGATTAGTGACCAGGGTTTTCAACTACAATAACTGCTTA    3633
AATCTATATATACCCTCCTCAATATCTGCTTAACTACATATAGATTCAAGTGTGT
TTTAAGATTAAAGTTGTAGTTTTTTATTCATGCTGATAATATAACTCTGTAGTT
AACCTTGTGTGAAATGTTTAACTTTATTTCATTTAAATTGCTGTTTCGTTCGTT
CCATTTAFGACAATGCCAAAATATGGAGAAACATCAT            3820
```

FIG. I-4(A)

FIG. I-4(B)

FIG. 1-5

## FIG. I-6(A)



## FIG. I-6(B)

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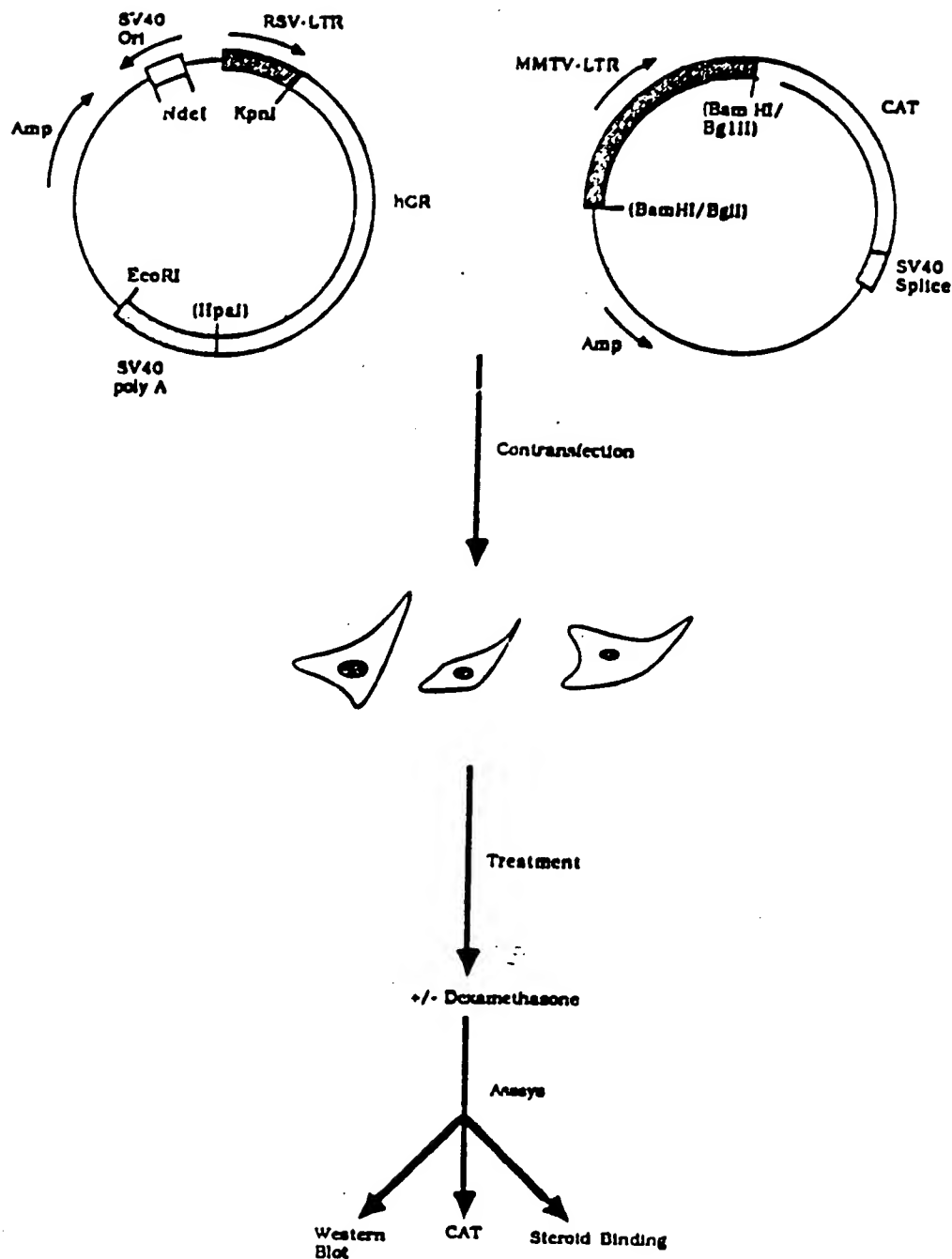


Schematic Representation of the hGR Functional Assay

# FIG. II-1

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FIG. II-2

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FIG. II-4(B)

pRShGRα (log pM)

FIG. II-4(A)

DEX (log M)

CAT ACTIVITY (% maximal induction)

FIG. II-5

FIG. III-1(A)

## FIG. III-1(B)-1

FIG. III-1(B)-2

```
            210                          220                          230
LysSerIleGlyHisLysProGluThrAspGluLeuProIleLeuIleGluLysIleThrGlyLysValAlaThrAsnAlaGln
AATTCAATGGGGCACAAGCCAGAACGAGGAATGGAAATCCAGTCAATCAAAACTGTAACGAAAGTGCATCGGCCACCAACGCCCAA  990

            240                          250                          260
GlySerHisTrpLysGluProLysPheLeuProGluAspIleGlyGlnAlaProLeuValAlaProGluGluGlyGlyLysValAsp
GCAGGCCACTGGAAGCAAAACCGAAATTCTGCCAGAAGACATTGGACAAGCACCAATAGTCGCTCCCGAAGGTGGAAGGTTGAC  1080

            270                          280                          290
LeuGluValAlaPheSerHisPheThrLysIleLeuIleThrProAlaIleLeuThrArgValValAlaAspPheAlaLysLysLeuProMetPheCysGlu
TTGGAAGCCTTCAGCCATTTTACAAAATCATCACCACCAGCAATTACCAGATGGTGGCAAAAGTTGCCTATGTTTTGTGAG  1170

            300                          310                          320
LeuProCysGluAspGlnIleIleLeuLeuLysGlyCysCysMetGluIleMetSerLeuArgAlaAlaValArgTyrAspProGluSer
CTGCCATGCGAAGACCAGATCATCCTGCTGAAATGGTGCTGCATGGAAATCATGTCCCTGCGTGCGCTAGACCGTAGACCCGGAAAGT  1260

            330                          340                          350
GluThrLeuThrLeuAsnGlyGluMetAlaValIleArgGlyHisLysTyrAspValValValAlaAspAlaIleArgAsp
GAACTTTAACCTGAATGGGGAAATGGCAGTAATAGGCACTGAAAAGTGGGATGTTGGG  1350

            360                          370                          380
LeuGlyMetSerLeuSerPheAsnLeuAspAspThrGluValAlaIleLeuLeuMetSerSerAspArgProGly
CTAGGCATGTCTCGTCTTCTTCAACCTGGATGACACTGAAGTCGCCATTCTCAGCTTCAGATCGCCCGGGG  1440

            390                          400                          410
LeuAlaCysValGluArgIleGluLysTyrGlnAspSerPheLeuLeuAlaPheLysHisTyrIleAsnTyrArgGlyHisHisValThr
CTCCCTGCGTGAGAGAATAGAAAAATACCAAGATAGTTCCTGCTGGCCTTCAAGCAATATCAATTACAGAAAAACCACCGTGACA  1530

            420                          430                          440
HisPheTrpProLysLeuLeuMetLysGluValThrAspLeuArgMetIleGlyAlaCysHisAlaSerArgPheLeuHisMetLysValGlu
CATTTTCGGCCAAAACTCCTGCATGAAGCTGACAGATCTGCGGAT... GCCAGCCGCTGCAGATGAAGGTGGA  1620

            450
CysProThrGluLeuPheLeuGluValPheGluAsp(End)
TGCCCAACAGAACTCCTCCCCCTTGTCCTGAAGTGTTCACATTAGTCACTGCATTCCTCCTATAATTCAAAAAAAAA  1710
```

FIG. III-2

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v-erbA
c-erbA
hGR
hER

```
 56 ... G . K . . T G Y M . . . T G . . . F F R P T . .    v-erbA
101 I . . V V . S . E . . S . C . H . Y G . . T . . F F K . A V    c-erbA
133 Y . . A . . . . N . Y . S . T H Y . . . S . C . . A F F K E . .    hGR
                                                                     hER

146 Q . . L H P I Y . C Y D G C . V . Q F I T . N Q . . L L . R    v-erbA
111 Q K N L H P S Y . C . K Y E G . . V . I D K . . T P . . E . . R R    c-erbA
150 C . . . . H N Y L . A G R N . . . . I D K . P K . . P A . G R    hGR
211 G . . . H N Y M . C P A T N . . . . I D K . N R . K S . . Q A . R    hER

 86 F K . C I . . G M A M D L V L . D S K . A K R K L I E E N    v-erbA
161 F Y K C I . . G M A T D L V L . D S K . A . K . I I E E N    c-erbA
478 Y R . C L Q . G M N L E A R K . - . . . T . K . K G . .    hGR
242 L Q . Y E . G M K G G I . . . R R . . G G . M L K H . . -    hER

125 R Q . R . K E E N I K S L Q . . P S P G A E E W E L I . .    v-erbA
191 R E K R . . R F E L Q K S I G H . P E P T D E E W E L I K T .    c-erbA
500 - I . Q A I T G V S Q E T S E . P G N K T I V P A T L P Q L    hGR
258 - K R Q . . D G E G R G E V . . A G D M R A A N L W P S P L    hER

156 - - - - - . . F A H R S T N A Q G S H W . Q R R K F L L . . I    v-erbA
221 - - - - - . . F A H V A T N A Q G S H W . K . K P K F L P . . I    c-erbA
529 - - - - - . . P T L V S L . . . . . . . . . . . . . . L . V    hGR
297 M I K R S . . N S L A L S L T A D Q M V S A . . . . . L L D    hER

181 G . . . . . H . . M L D G D K L - . L E . F S E - - F . K I I T    v-erbA
216 G . . . I . . A P E G G K V - . L E . F S H - - F . K I I T    c-erbA
549 I E P . . . A G Y D S S V P . . S T W . I M T T L N M L G G    hGR
322 A . . . I . . S E Y D P T R . . . S A . M M G L L . N . A D    hER

253 P A I T R V . Q R A K N L P . . S E L P C E . . I I L . K G    v-erbA
273 P A I T R V . D F A K K L P M F C E L P C E . . I I L . K G    c-erbA
569 R Q V I A A . K W K A . I P G . R N L H L D . . M T L . Q Y    hGR
352 R E L V H M . N . A K R V P G . . V D L T L H D . . M L L E C    hER

213 C C . M E I M S L R A A V R Y D P E S E . . - . L S G E M A    v-erbA
303 C C . M E I M S L R A A V R Y D P E S E . . - . L . N G E M A    c-erbA
599 S . M . . A F A L G W R S Y . P . S S A N L . L C F A P D L I    hGR
382 A . . L E I L M I G L V W R S M E H P V K - . . L F A P N L L    hER

266 V . . P E Q L . N G G L G V V S . . A - I F . . - - - - L . G K    v-erbA
331 V I . P G Q L . N G G L G V V S . A - I F . . - - - - L . G M    c-erbA
629 I N . . Q . M . P C M Y D Q . C K H M . . S S E L H R . . Q V    hGR
410 L . . . . N . G K - . C V E G M V E - . . . I F . . - - - M . L L    hER

289 S L S A - - - - . F N L D D T E V A L L Q A V L . M S S D - -    v-erbA
354 S L S S - - - - . F N L D D T E V A L L Q A V L . M S S D - -    c-erbA
659 S Y - - - - - . . E E Y L C M K T L L . L S S V P -    hGR
430 A . S R F R M M N L . Q G E E F V C L K S I I L L N S G V Y    hER

313 - - - R . G L I C V H K I E K . Q . . . . . . . E S Y L L A    v-erbA
378 - - - R P G L A C V E R I E K Y Q . . . . . . . D S F L L A    c-erbA
677 - K D . L K S . . E L F D E I R . . . . . M T Y I K E    hGR
460 T F L S S . L K S L . E K D M I H R V L D K I T D T L I H L    hER

333 F E . . . N . . R K H N I P H F W S K . . - L L . K V A D L . R    v-erbA
398 F E H . . I . . R K H M V . H F W P K . . - L L . K V T D L . R    c-erbA
697 L G K A I V K R E G N S S . . Q R F . Q L L . K L L D S . H    hGR
490 M A K A G L F L Q . . . . . . Q H Q R L A Q L L L I L S H I . .    hER

363 M I . A Y A A S . F L H M V E C P T E L ——— 19 residues    v-erbA
365 M I . A C H A S R F L H M V E C P T E L ——— 11 residues    c-erbA
. . . . E . . . . . . . . . . . . . . . . . . . ——— 48 residues    hGR
. . . H M . . . . . . . . . . . . . . . . . ——— 11 residues    hER
```
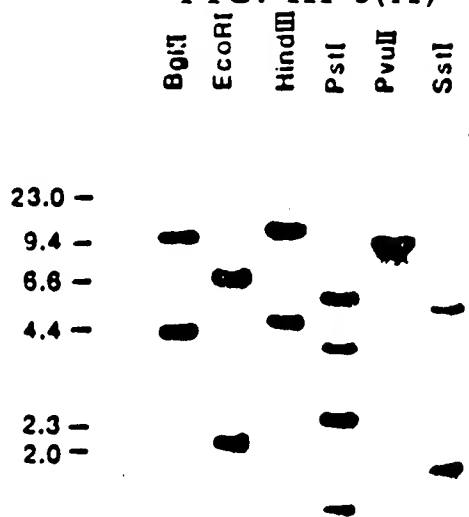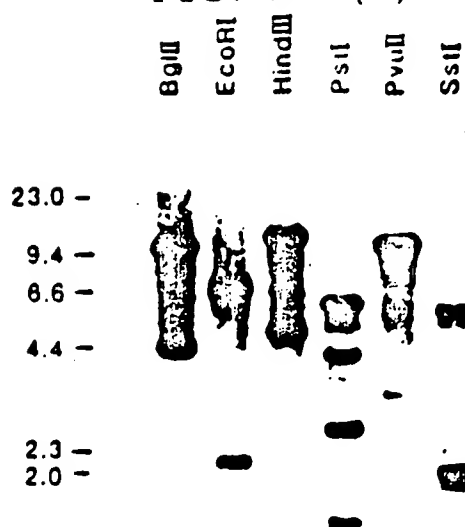
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FIG. III-3(A)

FIG. III-3(B)

FIG. III-3(C)

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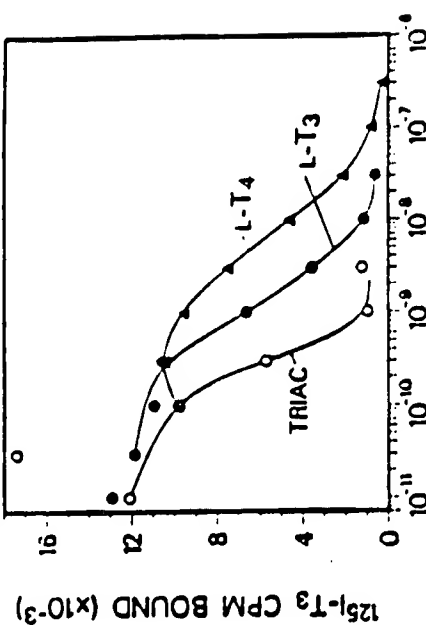FIG. III-4(B)

1 2 3 4

1

66kD—

45kD—

30kD—

FIG. III-4(A)

1 2 3 4 5

1

28S—

18S—

FIG. III-5(A)

FIG. III-5(B)

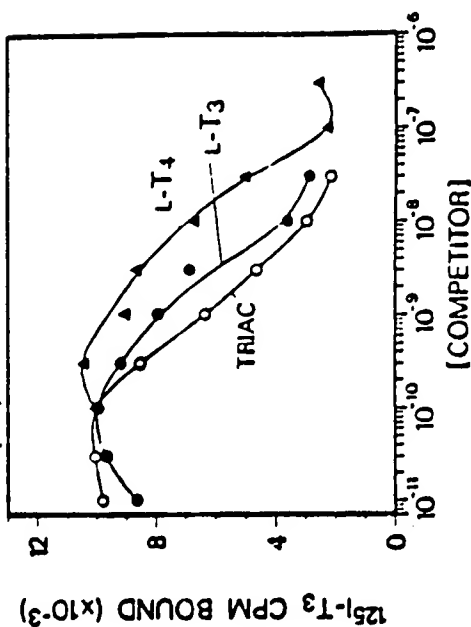FIG. III-5(C)

FIG. III-5(D)

FIG. III-6

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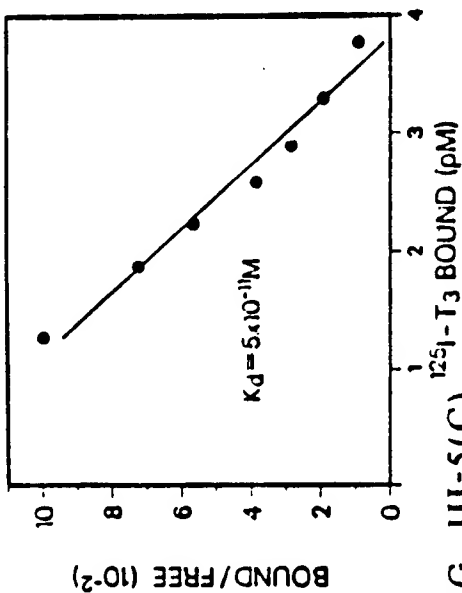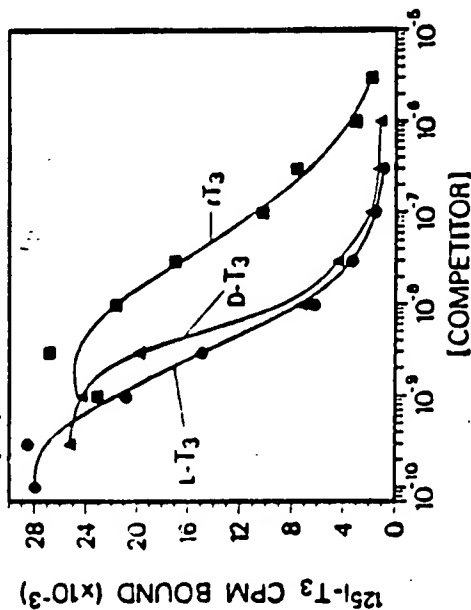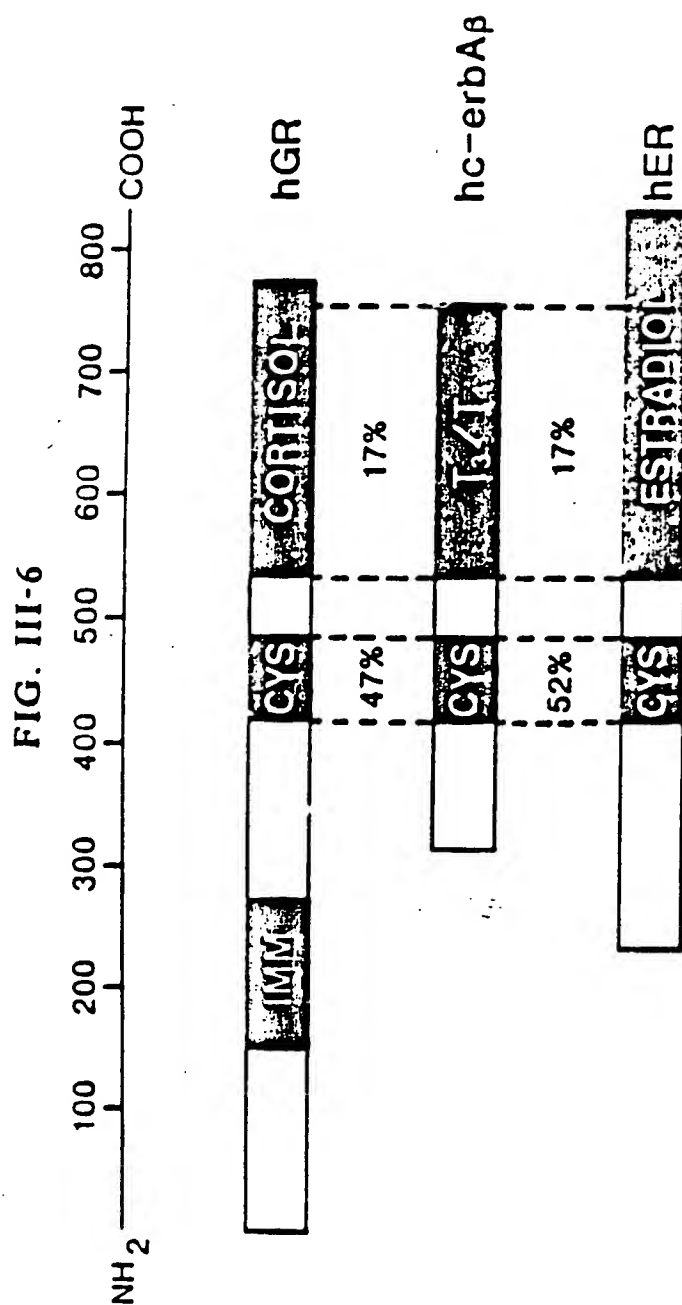
# FIG. III-7

```
                        10                                        20
GAATTGAAGTGAATGGAACAGAAGCCAAGCAAGGTGGAGTGTGGGTCAGACCCAGAGGAGAACAGTGCCAGGTCACCACATGGAAAGCGA          90
            MetGluGlnLysProSerLysValGluCysGlySerAspProGluGluAsnSerAlaArgSerProAsnGlyLysArg
              30                                       40                                      50
AAAAGAAAGAACGGCCAATGTTCCCTGAAAACCAGCATGTCAGGGTATATCCCTAGTTACCTGGACAAAGACGAGCAGTGTGTGTCGTGTGT         180
    LysArgLysAsnGlyGlnCysSerLeuLysThrSerMetSerGlyTyrIleProSerTyrLeuAspLysAspGluGlnCysValValCys
                   60                                      70                                      80
GGGGACAAGGCAACTGGTTATCACTACCGCTGTATCACTTGTGAGGGCTGCAAGGGCTTCTTTCGCCCGCACAATCCAGAAGAACCTCCAT         270
   GlyAspLysAlaThrGlyTyrHisTyrArgCysIleThrCysGluGlyCysLysGlyPhePheArgArgThrIleGlnLysAsnLeuHis
                            90                                     100                                      110
CCCACCTATTCCTGCAAATATGACAGCTGCTGTGTCATTGACAAGATCACCCGCAATCAGTGCCAGCTGTGCCGCTTCAAGAAGTGCATC         360
   ProThrTyrSerCysLysTyrAspSerCysCysValIleAspLysIleThrArgAsnGlnCysGlnLeuCysArgPheLysLysCysIle
                            120                                    130                                     140
GCCGTGGGCATGGCCATGGACTTGGTTCTAGATGACTCGAAGCGGGTGGCCAAGCGTAAGGTCGATTCAGCAGAACCGGGAGCGGCGGCGG         450
   AlaValGlyMetAlaMetAspLeuValLeuAspAspSerLysArgValAlaLysArgLysLeuIleGluGlnAsnArgGluArgArgArg
                            150                                    160                                     170
AAGGAGGAGATGATCCGATCACTGCAGCAGCGACCAGAGCCCACTCCTGAAGAGTGGGATCTGATCCACATTGCCACAGAGGCCCCATCGC         540
   LysGluGluMetIleArgSerLeuGlnGlnArgProGluProThrProGluGluTrpAspLeuIleHisIleAlaThrGluAlaHisArg
                            180                                    190                                     200
AGCACCAATGCCCAGGGCAGGCATTGGAAACAGAGGCGGAAATTCCTGCCCGATGACATTGGGCAGTCACCCATTGTGTCCATGCCGGAC         630
   SerThrAsnAlaGlnGlyArgHisTrpLysGlnArgArgLysPheLeuProAspAspIleGlyGlnSerProIleValSerMetProAsp
                            210                                    220                                     230
GGAGACAAGGTGGACCTGGAAGCCTTCAGCGAGTTTACCAAGATCATCACCCCGGGCATCACCCGTGTGGGTGGACTTTGCCAAAAAACTG         720
   GlyAspLysValAspLeuGluAlaPheSerGluPheThrLysIleIleThrProGlyIleThrArgValValAspPheAlaLysLysLeu
                            240                                    250                                     260
CCCATGTTCTCCGAGCTGCCCTTGCGAAGACCAGATCATCCTCCTGAAGGGGTGCTGCATGGAGATCATGTCCCTGCGGGCCGGCTGTCCGC         810
   ProMetPheSerGluLeuProCysGluAspGlnIleIleLeuLeuLysGlyCysCysMetGluIleMetSerLeuArgAlaAlaValArg
                            270                                    280                                     290
TACGACCCTGAGAGCCGACACCCTGACGCTGAGTGGGGAGATGGCTGTCAAGCGGGAGCAGCCCAAGAATGGCGGCCTGGGCGTAGTCTCC         900
   TyrAspProGluSerAspThrLeuThrLeuSerGlyGluMetAlaValLysArgGluGlnLeuLysAsnGlyGlyLeuGlyValValSer
                       300                                     310                                     320
GACGCCATCTTTGAACTGGGCAAGTCACTCTCTGCCCTTTAACCTGGATGACACGGAAGTGGCTCTGCTGCCAGGCTGTGCTGCTAATGTCA         990
   AspAlaIlePheGluLeuGlyLysSerLeuSerAlaPheAsnLeuAspAspThrGluValAlaLeuLeuGlnAlaValLeuLeuMetSer
                            330                                    340                                     350
ACAGACCGCTCGGGCCTGCTGTGTGTGGACAAGATCGAGAAGAGTCAGGAGGCGTACCTGCTGGCGTTCGAGCACTACGTCAACCACCGC        1080
   ThrAspArgSerGlyLeuLeuCysValAspLysIleGluLysSerGlnGluAlaTyrLeuLeuAlaPheGluHisTyrValAsnHisArg
                            360                                    370                                     380
AAACACAACATTCCGCACTTCTGGCCCAAGCTGCTGATGAAGGAGAGAGAAGTGCAGAGTTCGATTCTGTACAAGGGGGCAGCGGCAGAA        1170
   LysHisAsnIleProHisPheTrpProLysLeuLeuMetLysGluArgGluValGlnSerSerIleLeuTyrLysGlyAlaAlaAlaGlu
                            390                                    400                                     410
GGCCGGCCGGGCGGGTCACTGGGCGTCCACCCGGAAGGACAGCAGCTTCTCGGAATGCATGTTGTTCAGGGTCCGCAGGTCCGGCAGCTT        1260
   GlyArgProGlyGlySerLeuGlyValHisProGluGlyGlnGlnLeuLeuGlyMetHisValValGlnGlyProGlnValArgGlnLeu
                            420                                    430                                     440
GAGCAGCAGCTTGGTGAAGCGGGAAGTCTCCAAGGGCCGGTTCTTCAGCACCAGAGCCCGAAGAGCCCGCAGCAGGCTCTCCTGGAGCTG        1350
   GluGlnGlnLeuGlyGluAlaGlySerLeuGlnGlyProValLeuGlnHisGlnSerProLysSerProGlnGlnAlaLeuLeuGluLeu
                            450                                    460                                     470
CTCCACCGAAGCGGAATTCTCCATGCCCGAGCGGTCTGTGGGCAAGACGACAGCAGTGAGGCGGACTCCCCGAGCTCCTCTGAGGAGGAA        1440
   LeuHisArgSerGlyIleLeuHisAlaArgAlaValCysGlyGluAspAspSerSerGluAlaAspSerProSerSerSerGluGluGlu
                            480                                    490
CCGGAGGTCTGCGAGGACCTGGCAGGCAATGCAGCCTCTCCCTGAAGCCCCCCAUAAGGCCGATGGGGAAGGAGAAGGAGTGCCATACCT        1530
   ProGluValCysGluAspLeuAlaGlyAsnAlaAlaSerProEnd

TCTCCCAGGCCTCTGCCCCAAGAGCAGGAGGTGCCTGAAAGCTGGGAGCGTGGGCTCAGCAGGGCTGGTCACCTCCCATCCCGTAAGACC        1620
ACCTTCCCTTCCTCAGCAGGCCAAACATGGCCAGACTCCCTTGCTTTTTGGTGTGTAGTTCCCTCTGCCTGGGATGCCCTTCCCCCTTTC        1710
TCTGCCTGGCAACATCTTACTTGTCCTTTGAGGCCCCAACTCAAGTGTCACCTCCTTCCCCAGTTCCCCCAGGCAGAAATAGTTGTCTGT        1800
GGCTTCCTTGGTTCATGCTTCTACTGTGACAGTTATCTCACTGTTTTATAATTAGTCGGGCATGAGTCTGTTTCCCAAGCTAGACTGTGTC        1890
CGAATCATGTCCTATCCCCAGTGCCCGGTGCCAGGGCCTGGCATAGAGTAGGTACTCCATAAAAGGTGTGTTGAATTGAAAAAAAAAAA        1980
```

FIG. IV-1(A)   FIG. IV-1(B)   FIG. IV-1(C)

FIG. IV-1(D)

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FIG. IV-2(A)

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FIG. IV-2(B)-1

FIG. IV-2(B)-2
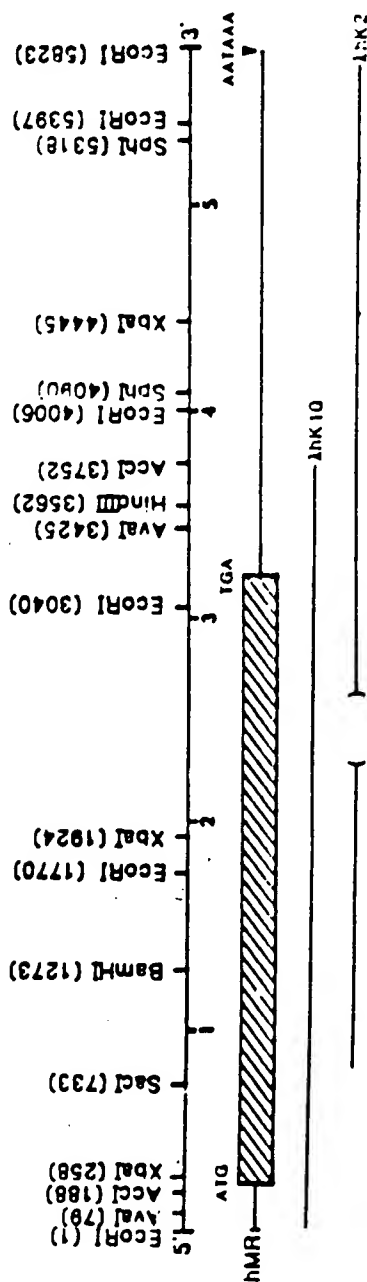
## FIG. IV-3

```
hMR  548  SSFPPVNILVESWKSHGDLSSRRSDGYPVLEYIPENVSSSTLRSVSTGSS
                         |                         |||
hGR  374  NLTSLGTLNFPGRTVFSNGYSSPS........MRPDVSSPPSSSTATTG
                    ┌─DNA──►
hMR  598  RPSKICLVCGDEASGCHYGVVTCGSCKVFFKRAVEGQHNYLCAGRNDCII
          | | ||||| |||||||||| ||||||||||||||||||||||||||||
hGR  416  PPPKLCLVCSDEASGCHYGVLTCGSCKVFFKRAVEGQHNYLCAGRNDCII
                       ◄──DNA──┐
hMR  648  DKIRRKNCPACRLQKCLQAGMNLGARKSKKLGKLKGIHEEQPQQQQPPPP
          ||||||||||||| | ||||||||||||| ||| ||  |
hGR  466  DKIRRKNCPACRYRKCLQAGMNLEARKTKKKIKGIQQATTGVSQ......
                                        ┌──STEROID──►
hMR  698  PPPPQSPEELTTYIAPAKEPSVNTALVPQLSTISRALTPSPVMVLENIEP
                              |||           |||  | || |||
hGR  510  ................ETSENPGNKTIVPATLPQLTPTLVSLLEVIEP
hMR  748  EIVYAGYDSSKPDTAENLLSTLNRLAGKQMIQVVKWAKVLPGFKNLPLED
          | ||||||| ||            ||| | |  |  ||||| || || |
hGR  542  EVLYAGYDSSVPDSTWRIMTTLNMLGGRQVIAAVKWAKAIPGFRNLHLDD
hMR  798  QITLIQYSWMCLSSFALSWRSYKHTNSQFLYFAPDLVFNEEKMHQSAMYE
          | || ||||||| |   ||| ||||      | |||||| ||   ||
hGR  592  QMTLLQYSWMFLMAFALGWRSYRQSSANLLCFAPDLIINEQRMTLPCMYD
hMR  844  LCQGMHQISLQFVRLQLTFEEYTIMKVLLLLSTIPKDGLKSQAAFEEMRT
          | |  |||   |||  |||-|| ||||||  |||||||||  | | |
hGR  642  QCKHMLYVSSELHRLQVSYEEYLCMKTLLLLSSVPKDGLKSQELFDEIRM
hMR  898  NYIKELRKMVTKCPNNSGQSWQRFYQLTKLLLDSMHDLVSDLLEFCFYTFR
          |||||  |  |   || ||||||||||||||||||||||  | || || ||
hGR  692  TYIKELGKAIVKREGNSSQNWQRFYQLTKLLLDSMHEVVENLLNYCFQTF.
                         ◄──STEROID──┐
hMR  948  ESHALKVEFPAMLVEIISDQLPKVESGNAKPLYFHRK  984
          |||  ||| ||| || | ||   ||| ||| ||| |||
hGR  741  LDKTMSIEFPEMLAEIITNQIPKYSNGNIKKLLFHQK  777
```

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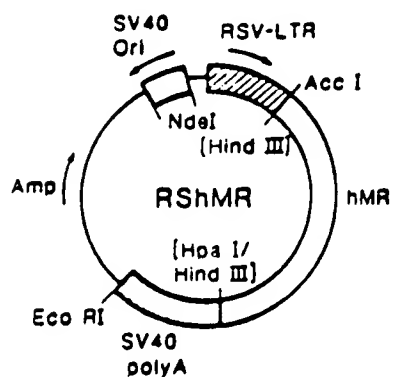
FIG. IV-4(A)

FIG. IV-4(B)
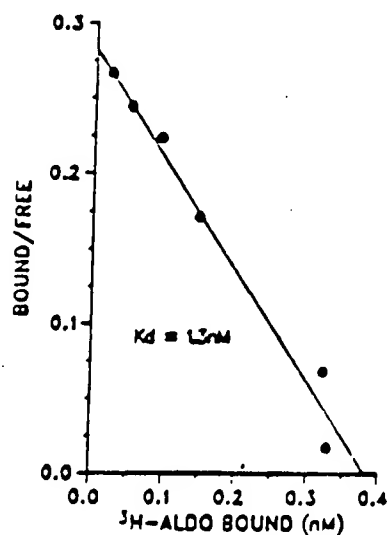
FIG. IV-4(C)
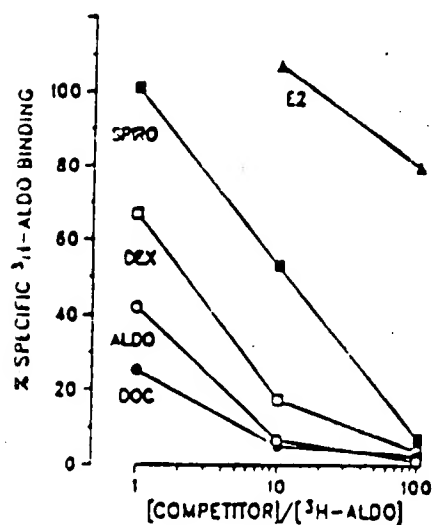
FIG. IV-4(D)
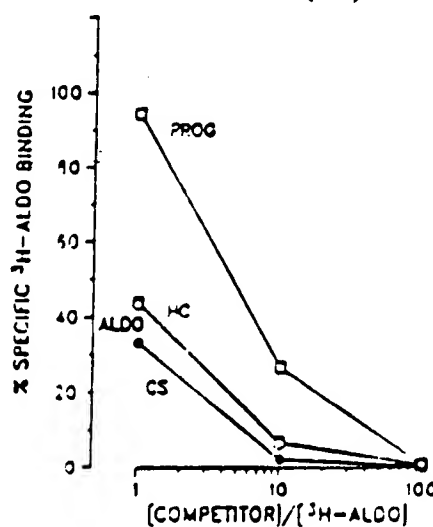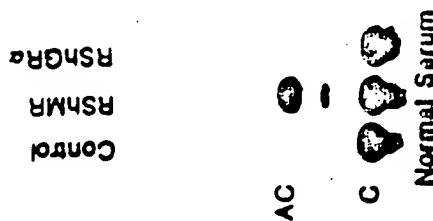
FIG. IV-5(C)

FIG. IV-5(B)

FIG. IV-5(A)
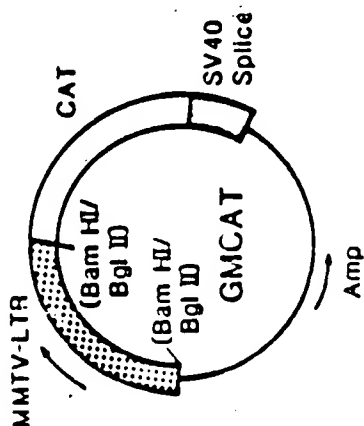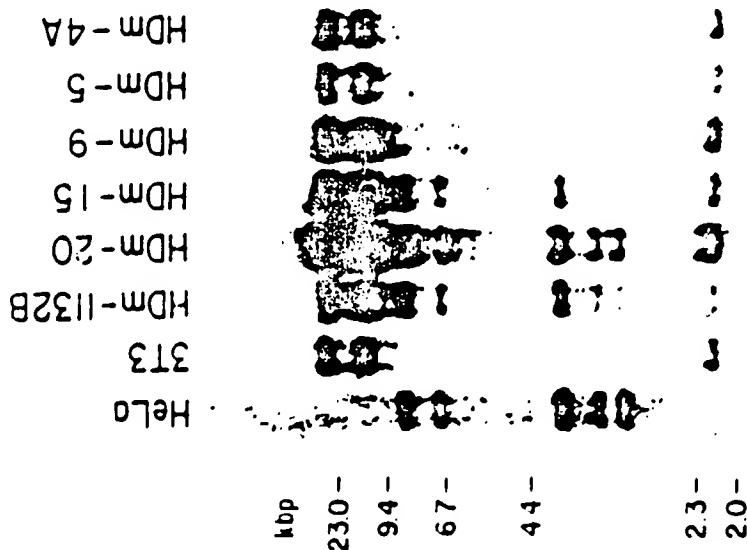
FIG. IV-7



HDm-4A
HDm-5
HDm-9
HDm-15
HDm-20
HDm-1132B
3T3
HeLa

kbp
23.0 —
9.4 —
67 —
44 —
2.3 —
2.0 —

FIG. IV-6



Muscle
Spleen
Liver
Heart
Pituitary
Hippocampus
Brain
Gut
Kidney

28S --
18S --

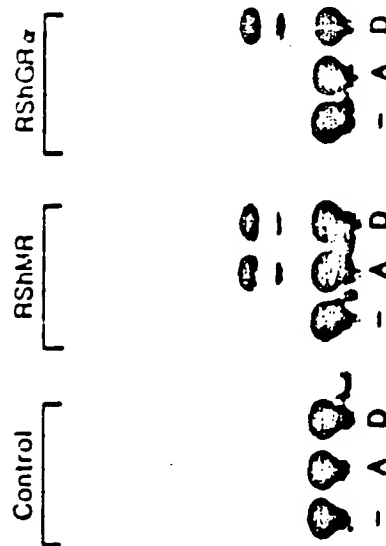FIG. IV-8
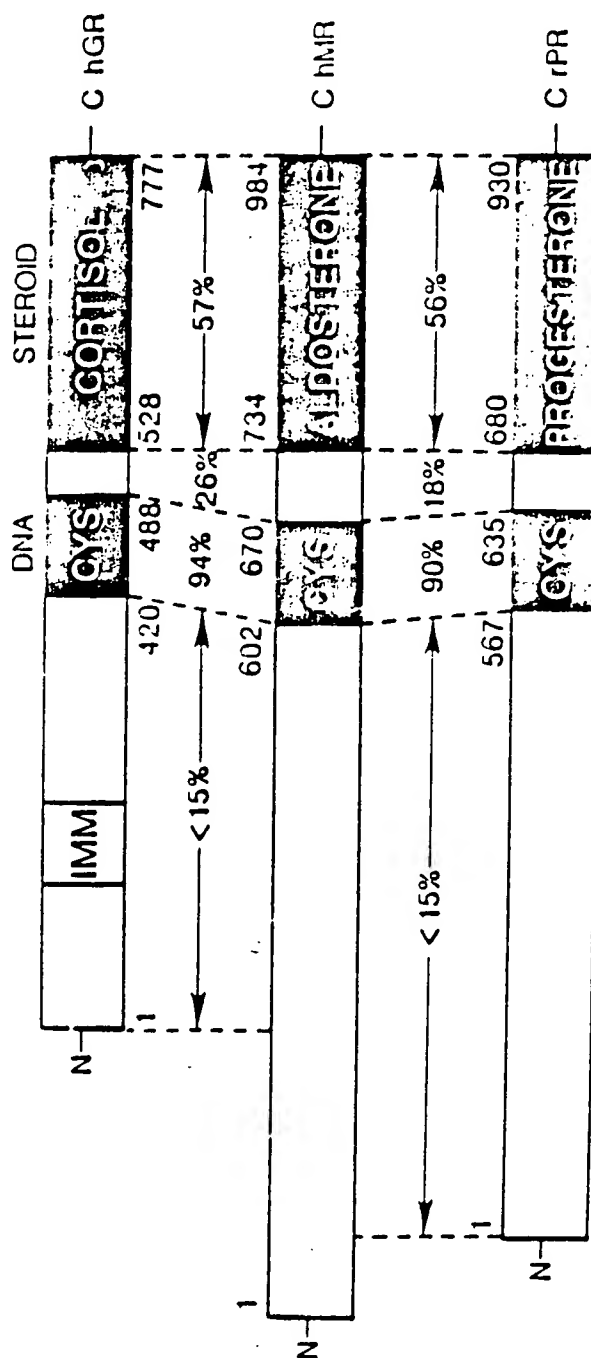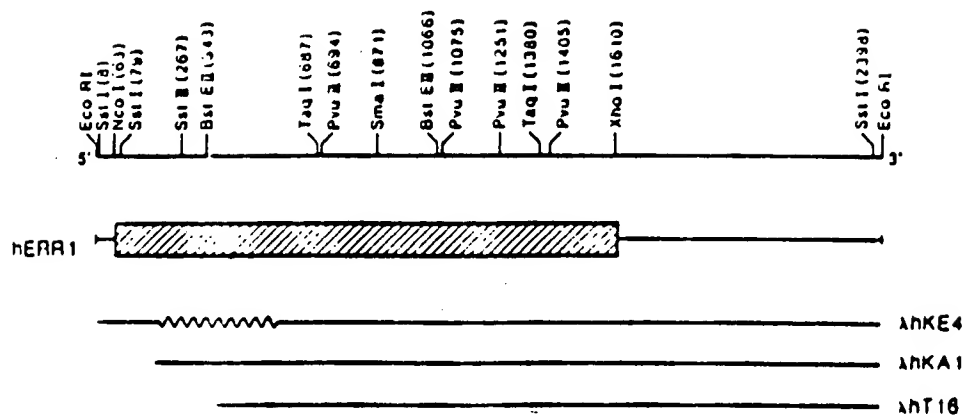
FIG. V-1(A)



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## FIG. V-1(B)-1

```
                                                                    1
                                                       MetGlyLeuGluMetSerSerLysAsn
  1  AGCTCACAGCCAAGTCCAGGCTAGAGGTAGAAACGTGAGAGCCCCACGGCTGGGGAAGATTGCCATGGGATTGGAGATGAGCTCCAAGGAC

      11                        21                        31
      SerProGlySerLeuAsoGlyArgAlaTrpGluAsoAlaGlnLysProGlnSerAlaTrpCysGlyGlyArgLysThrArgValTyrAla
  91  AGCCCTGGCAGTCTGGATGGAAGAGCTTGGGAAGATGCTCAGAAACCACAAAGTGCCTGGTCCCGTCGGACGAAAACCAGAGTGTATGCT

      41                        51                        61
      ThrSerSerArgArgAlaProProSerGluGlyThrArgArgGlyGlyAlaAlaSerArgProGluGluAlaAlaGluGluGlyProProAla
 181  ACAAGCAGCCGCCGGCGCCCGCCCAGTGAGGGGACGCGGCGCGTGGGGCGGCCGCGCCCCAGGAGCCGGCCGAGGAGGGCCCCCCCGCG

      71                        81                        91
      AlaProGlySerLeuArgHisSerGlyProLeuGlyProHisAlaCysProThrAlaLeuProGluProGlnValThrSerAlaMetSer
 271  GCCCCCGGCTCACTCCGTCACTCCGGGCCGCTCGGCCCCCATGCCTGCCCGACCGCGCTGCCGGAGCCCCAGGTGACCAGCGCCATGTCC

      101                       111                       121
      SerGlnValValGlyIleGluProLeuTyrIleLysAlaGluProAlaSerProAsnSerProLysGlySerSerGluThrGluThrGlu
 361  AGCCAGGTCGTGGGCATTGAGCCTCTCTACATCAAGGCAGACCCGGCCAGCCCTGACAGTCCAAAGGGTTCCTCGGACACAGAGACCGAG

      131                       141                       151
      ProProValAlaLeuAlaProGlyProAlaProThrArgCysLeuProGlyHisGluGluGluAsoGlyGluGlyAlaGlyProGly
 451  CCTCCTGTGGCCCCTGCCCCCTGGTCCAGCTCCCACTCGCTGCCTCCCAGGCCACAAGGAAGAGGAGGATGGGGAGGGGGGCTCGGGCCTCGGC

      161                       171                       181
      GluGlnGlyGlyGlyLysLeuValLeuSerSerLeuProLysArgLeuGlnCysLeuValIleCysGlyAspValAlaSerGlyTyrHisTyrGly
 541  GAGCAGGGCGGTGGGAAGCTCGTGCTCAGCTCCCTGCCCAAGCGCCTCTGCCTGGTCTGTGGGGACGTGGCCTCCGGCTACCACTATGGT

      191                       201                       211
      ValAlaSerCysGluAlaCysLysAlaPhePheLysArgThrIleGlnGlySerIleGluTyrSerCysProAlaSerAsnGluCysGlu
 631  GTCGCCATCCTGTGAGCCCTGCAAAGCCTTCTTCAAGAGGACCATCCAGGGGAGCATCGAGTACAGCTGTCCCGCCCTCCAACGAGTGTGAG

      221                       231                       241
      IleThrLysArgArgArgCysAlaCysGlnAlaCysArgPheThrLysCysLeuArgValGlyMetLeuLysGluGlyValArgLeuAsp
 721  ATCACCAACCGGAGACCCAAGGCCTGCCAGCCCTGCCGCTTCACCAAGTGCCTGCGGGTGGGCATGCTCAACGAGGGAGTGCGCCTCGAC

      251                       281                       271
      ArgValArgGlyGlyArgGlnLysTyrLysArgArgProGluValAspProLeuProProPheProGlyProPheProAlaGlyProLeuAla
 811  CGCGTCCCGGGTGGGCGGCAGAAGTACAAGCGGCGGCCCGGAGGTGGACCCACTGCCCTTCCCGGGCCCCTTCCCTGCTGGGCCCCCTGGCA

      281                       291                       301
      ValAlaGlyGlyProArgLysThrAlaAlaProValAsnAlaLeuValalSerHisLeuLeuValValGluProGluLysLeuTyrAlaMet
 901  GTCGCTGGAGGCCCCCCGGAAAGACAGCAGCCCCAGTGAATGCACTGGTGTCTCATCTGCTGGTGGTTGAGCCTGAGAAGCTCTATGCCATG
```

## FIG. V-1(B)-2

```
        311                         321                         331
    ProAspProAlaGlyProAspGlyHisLeuProAlaValAlaThrLeuCysAspLeuPheAspArgGluIleValValThrIleSerTrp
991 CCTGACCCCGCAGGCCCTGATGGCACCTCCCAGCCGTGGCTACCCTCTGTGACCTCTTTGACCCGAGAGATTGTGGTCACCATCAGCTGG

        341                         351                         361
     AlaLysSerIleProGlyPheSerSerLeuSerLeuSerAspGlnMetSerValLeuGlnSerValTrpMetGluValLeuValLeuGly
1081 GCCAAGAGCATCCCAGGCTTCTCATCGCTGTCGCTGTCTGACCAGATGTCAGTACTGCAGAGCGTGTGGATGGAGGTGCTGGTGCTCGGT

        371                         381                         391
     ValAlaGlnArgSerLeuProProLeuGlnAspGluLeuAlaPheAlaGluAspLeuValLeuAspGluGluGlyAlaArgAlaAlaGlyLeu
1171 GTGGCCCAGCGCCTCACTGCCCACTGCAGGATGAGCTGGCCTTCGCTGAGGACTTAGTCCTGGATGAAGAGGGCGCCACGGGCAGCTGGCCTG

        401                         411                         421
     GlyHisLeuGlyValAlaAlaLeuLeuGlnLeuGluValArgArgLeuGlnAlaLeuLeuArgLeuGluValLeuArgGlnValTyrValLeuLeuLysAlaLeu
1261 GGGCAACTGGGGCGCTGCCCTGCTGCAACTAGTGCGGCGGCCTGCAGGCCCTGCCGCTCGAGCGAGGAGTATGTTCTACTAAAGGCCTTG

        431                         441                         451
     AlaLeuGlnIleAlaSerAspSerValHisIleIleGluAspGluLeuProArgLeuTrpSerSerCysGlyLysLeuGlnHisGluValAlaLeuLeuGlu
1351 GCCCTTGCCAATTCAGACTCTGTGCACATCGAAGATGAGCCGAGGCTGTGGAGCAGCTGCCGACAAGCTCCTGCACGAGGCCCTGCTGGAG

        461                         471                         481
     TyrGluAlaGlyArgAlaGlyProGlyGlyGlyAlaGluArgArgArgAlaGlyArgLeuLeuLeuLeuThrLeuProLeuGluArgGlnThr
1441 TATGAAGCCGGCCGGGCTGGCCCCGGAGGGGGTGCTGAGCGGCGGCGGGCGGGCAGGCTGCTGCTCACGCTACCGGTCCTCCGCCAGACA

        491                         501                         511
     AlaGlyLysValLeuGluAlaHisPheTyrGlyValLysLeuGluGlyLysValIleProMetHisLysLeuProPheLeuGluGluMetLeuGluAlaMet
1531 GCCGGCAAAGTGCTGGCCCATTTCTATGGCGTGAAGCTCGAGGGCAAGGTGCCCATGCACAAGCTGTTCTTGGAGATGCTCGAGGCCATG

        521
     MetAspSTOP
1621 ATGGACTGAGGCAAGGGGTCGGACTGGTGCGGGGTTCTGGCCAGGACCTGCCTAGCATGGGGTCAGCCCCCAAGGGCTGGGGCGGAGCTCGGG

1711 TCTCGGCAGTGCACAGCCTGCTGGCCAGGGCCAGGGCTAATGCCATCAGCCCCTGGGAAACAGGCCCCACGCCCTCTCCTCCCCCTCCTAGG
1801 GGGTGTCAGAAGCTGGGAACGTGTGTCCAGGCTCTGGGCACAGTGCTGCCCCTTGCAAGCCATAACGGTGCCCCCAGAGTGTAGGGGGCC
1891 TTGCGGAAGCCATAGGGGGCTGCACGGGATGCGTGGGAGGCAGAAACCTATCTCAGCGGACGGAAGGGGATGGAGGCCAGAGTCTCCCAGT
1981 GGGTGATGCTTTTGCTGCTGCTTAATCCTACCCCCTCTTCAAAGCAGAGTGGGACTTGGAGAGCAAAGGCCCATGCCCCCTTCGCTCCTC
2071 CTCTCATCATTTGCATTGGGCATTAGTGTCCCCCCTTGAAGCAATAACTCCAAGCAGACTCCAGCCCCTGGACCCCTGGGGTGGCCAGCG
2161 CTTCCCCATCAGCTCCCAACGAGCCTCCTCAGGGGGTAGGAGAGCACTGCCTCTATGCCCTGCAGAGCAATAACACTATATTTATTTTTG
2251 GGTTTGCCCAGGCAGCCGCAGGGACATGGGGCAAGCCACGGCCCAGAGCCCTTGGCTGTACAGAGACTCTATTTTAATGTATATTTGCTG
2341 CAAAGAGAAACCGCTTTTGGTTTTAAACCTTTAATGAGAAAAAAATATATAATACCGAGCTCAAAAAAAAAAAAAAAAAAAAAAAAAAAAA
```

FIG. V-2(A)



λ hH3

hERR2
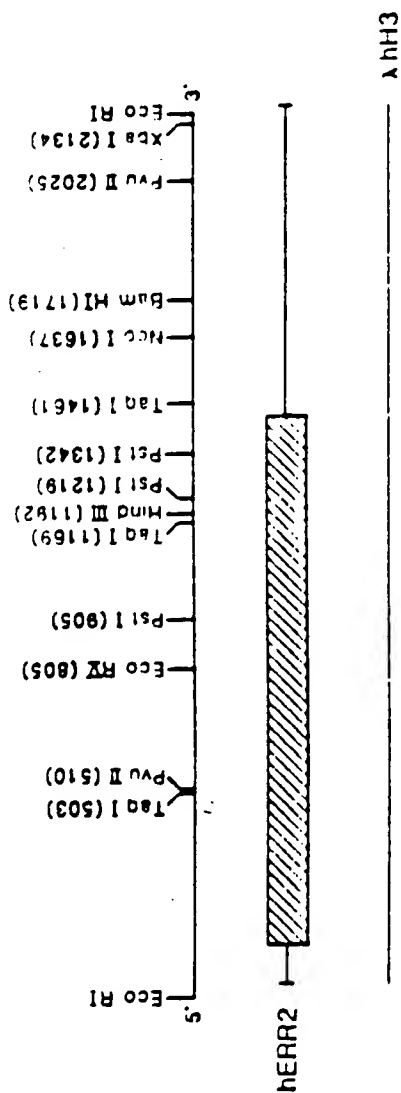
## FIG. V-2(B)-1

1    CTCCTCCAACTGGCAATGCTAAACGGGACTACATGACGTGTCCGAACTCTGCATCCCGGACCCCCTCGCCTACCACAACCAGTAGGTTG

         MetSerSerGluAspArgHisLeuGlySerCysGlySerPheIleLysThrGluProSerSerGlyIle
91   CTGAACCGAATGTCGTCCGAAGACGFCACCTGGGCTCTAGCTGCGGGCTCCTTCATCAAGACGGAGCCATCTAGCCCATCCTCGGGCATT

         AspAlaLeuSerHisHisSerProSerGlySerSerAspAlaSerGlyGlyPheGlyPheGlyMetAlaLeuGlyThrHisIleAlaAsnGlyLeuAsp
181  GATGCCCTCAGCCACCACAGCCCCAGCGGCTCGTCGGACGCCAGCGGTGGCTTTGGCATGGCCCTGGGCACCCACGGTCTGGAC

         SerProProMetPheAlaAlaGlyAlaGlyLeuGlyGlyAsnProCysArgLysSerTyrGluAspCysThrSerGlyIleMetMetGluAspSer
271  TCTCCCGCTAIGTTCGCCGGTGCCGGGGCTGGAGGCAACCCGGTGCAGAAGAGCTACGAGGACTGTACAGGGATCATGATGGAGGACTCG

         AlaIleLysCysGluTyrMetLeuAsnAlaIleProLysArgLeuCysLeuValCysGlyAspIleAlaSerGlyTyrHisTyrGlyVal
361  GCCATCAAGTGCGAGTACATGCTTAACGCCATCCCCAAGCGCCTGTGCCTCGTGTGCGGGGACATTGCTTCTCCTACCACTATGGCGTG

         AlaSerCysGluAlaCysLysAlaPhePheLysArgThrIleGlnGlyAsnIleGluTyrSerCysProAlaThrAsnGluCysGluIle
451  GCCTCCTGCGAGGCTTGCAAGGCGTTCTTCAAGAGAACCATTCAAGGAAACATCGAATACAGCTGCCCTGCCACCAACGAGTGTGACATC

         ThrLysArgArgArgLysSerCysGlnAlaCysArgPheMetLysCysLeuLysValGlyMetLeuLysGluGlyValArgLeuAspArg
541  ACCAAACGGAGGCGGAAGTCCTGTCAGGCCTGTCGCTTCATGAAATGCCTCAAGGTGGGCATGCTGAAGGAAGGCGTCCGGCTTGACCGG

         ValArgGlyGlyArgGlnLysTyrLysArgArgLeuAspSerGluAsnSerProTyrLeuAsnProGlnIleSerProProAlaLysLys
631  GTGCGAGGCGGCCGCCAGAAGTACAAGAGACGGCTGGATTCGGAGAACAGCCCCTACCTAGCTTACACAGATTCCCCGCCTGCTAAAAG

         ProLeuThrLysTyrIleValSerTyrLeuLeuValAlaGluProAspLysLeuTyrAlaMetProProCy·AspAspValProGluGlyAspIle
721  CCATTGACTAAGAATGTCTCGTATCTACTGGTGGCCGAGCCGGACAAGCTGTACGCTATGCCTCCCGACGATGTGCCCGAAGGGGATATC

## FIG. V-2(B)-2

```
                          241
                          LysAlaLeuThrThrLeuCysAspLeuAlaAspArgGluLeuValIlePheLeuIleSerTrpAlaLysHisIleProGlyPheSerAsnLeu
                    811   AAGGCCCTGACCACICTCIGACITGGACACGGGAGCITGIGTICCATTAGTCCAAGCACCATCCAAGGITICCAACCTG

                                 251                          261
                          ThrLeuGlyAspGlnmetSerLeuLeuGlnSerAlaTrpmetGluIleLeuIleLeuGlyIleValIleTyrArgSerLeuProTyrAspAsp
                    901   ACACTCGGGGACCAGATAGCCTGCTGCAGTCGCCIGATGGAGATCCTCATCCTGGGCATCGTGTCACGGTCCCTATGATGAC

                                 271                          281
                          LysLeuAlaTyrAlaGluAspTyrIleLeuAspIleSerGluGlnHisSerArgLeuValAlaIriLeuIleSerGluTyrAlaIleLeuGlnLeu
                    991   AAGCTGGCATACGCCGGAGACTACATATCACTGAGCAGCAACACTCICCCTGGTGGCTATATTGTGGAGCCATCTTGCAGGTC

                                 291                          301
                          ValArgTyrLysValIleLysGluLeuGlyProheValIleuLysAlaLeuAlaLeuAlaAsnSerAspSermetTyrIle
                    1081  GTACCAGGTACAAGAAGTCAAGTGGAAGAAGAGTTGTGATGCTGAAGGCCCTGGCCCTGCCAACTCAGATTCAATGTACAATC

                                 311                          321
                          GluAsnLeuGluAlaValGlnLysLeuLeuGlnAspLeuLeuGluAlaGluAsnTyrGluLeuSerGlnArgHisGluGluProArg
                    1171  GAGAACCTGGAGGCTGTGCAGAAGCTTCAGGACCTGCTGCAGGACTATGAGCAGCCCATGAGGAGCCCALGG

                                 331                          341
                          ArgAlaGlyLysLeuLeuLeuThrLeuProLeuLeuArgGlnThrAlaIleAlaLysValGlnHisPheTyrSerValLysLeuGlnGly
                    1261  AGGGCCGGGAAGTGCTIGTTGACICGCCCTCCTGCGGCAGACAGCAACAAGGTGCCAGCATTTACAGTGAAGTTCAGGGC

                                 421                          431
                          LysValPrometHisLysLeuPheLeuGluValLeuGluAlaLysValGlu***
                    1351  AAGGTGCCCATGCACAAGTCTTCCTGGAAGTCTGAGGCCAAGGTGIATGCCCCCGCATGAGAGAGGATGGACACGGATCCACATGGA

                    1441  CACTTCCACGCGCCACCACCTCCACTTCTGCACACCTGCATCGAGCGCCTCGAGCTGTCCATCGAGCAGACAAGGGTTICTTCGTTCGTGGCCAT
                    1531  GTGCACACICTGCGGGGGCCAGCAGGATGGGAGATCTGAAGCTTCCTTAGCGTGGGGCTCATGTGACCGAGTTICTTGGTAT
                    1521  TTTTTTTTCCTTCTCCATCGGACCAGTCTAAGCTGCCCGCCGCGCTCCTTCCTGGGCTAGCCTGCAGCCACGGAGGAGCATCCCTT
                    1711  CCTGCAAGGATCCATTTCTGGACCACTCCATATTAGCACCTGAGCTACCTGCAGTAAGTGCCCAACCCCAAGACACT
                    1801  TAGATTGGGTCCTCTCAAGGTGITGGTATCACAGAGGCAGGCCCTTGAACAGGAGCTCTCAGTAGCTGTCTGCTCTCTGGGCTCTGTIGCCT
                    1891  CCTCAGTCTAGGCTCTGTCGTCCCCCCTTCCGCCCTACCCTGGTTTCTGTCCTATGTCTCAGTGGCTATCAGGAATACACCACATCC
                    1981  CACCACTAAGAGGCCGCCGTCCTCTCTACCCACCCTTCCGTTCTGTTTCCTATCGCCTATICGCTTGGCATATGGGCCATGAGGGTCTAGAAACCTATCAA
                    2071  AGAAAAGAGTTGGGTCCTATACCCACCCTTCCGTTCTGTTTCCTATCGCCTATICGCTTGGCATATGGGCCATGAGGGTCTAGAAACTATCAA
```

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```
174 L C L V C G D V A S G Y H Y G V A S C E A     A F F K R     I   hERR1
102 L C L V C G D I A S G Y H Y G V A S C E A     A F F K R     I   hERR2
184 Y C A V C N D Y A S G Y H Y G V W S C E G C   A F F K H   S I   hER
420 L C L V C S D E A S G C H Y G V L T G G S K V   F K R   A V     hGR

204 Q G S I C Y S C F A S N E C E I T K R R R K A C Q A C R F T     hERR1
132 Q G N I E Y S C P A T N E C E I T K R R R K S C Q A C R F M     hERR2
214 Q G W N D Y M C P A T N Q C T I D K R R R K S C Q A C R L R     hER
450 E G Q H N Y L C A G R N D C I I D K I R R K N C P A C     Y R   hGR

234 K C L R V G M L K E G V R L D R V R G G R Q K Y K R R P E V     hERR1
 32 K C L K V G M L K E G V R L D R V R G G R Q K Y K R R E D S     hERR2
244 K C Y E V G M M K G G I R K D R R G G R M L K H K T O R D D     hER
480 K C L Q A G M N L E A R K - - - - T K K K I K G I Q Q A T T     hGR

264 D P L P F P G P F P A G P L A V A G G P R K T - - - - - - -     hERR1
192 E N S P Y L S L Q I S P P A - - - - - - - - - - - - - - - -     hERR2
274 G E G R G E V G S A G D M R A A N L W P S P L M I K R S K K     hER
506 G V S Q E T S E N P G N K T I V P A I L - - - - - - - - - -     hGR

288 - - - - - A A P V N A L V S H L L V V E P E K L Y A M P D P     hERR1
205 - - - - - K K P L T K I V S Y L L V A E P D K L Y A M P P Q     hERR2
304 N S L A L S L T A D Q M V S A L L D A E P P I L Y S E Y D P     hER
528 - - - - - P Q L T P T L V S L L E V I E P E V L Y A G Y D S     hGR

313 A G P D G H L P A V A T L C D L F D R E I V V T I S W A K S     hERR1
231 D V P E G D I K A L T T L C D L A D R E L V F L I G W A K H     hERR2
334 T R P F S E A S M M G L L T N L A D R E L V H M I N W A K R     hER
551 S V P D S T W R I M T T L N M L G G R Q V I A A V K W A K A     hGR

343 I P G F S S L S L S D Q M S V L Q S V W M E V L V L G V A Q     hERR1
261 I P G F S N L T L G D Q M S L L Q S A W M E I L I L G I V Y     hERR2
364 V P G F V D L T L H D Q V H L L E C A W L E I L M I G L V W     hER
581 I P G F R N L H L D D Q M T L L Q Y S W M F L M A F A L G W     hGR

373 R S L P L Q D E - - L A F A E D L V D E E G A R - - - A A     hERR1
291 R S L P Y D D K - - L A Y A E D Y I M D E E H S R - - - L V     hERR2
394 R S M E H P V K - - L L F A P N L L L D R N Q G K - - C V E     hER
611 R S Y R Q S S A N L L C F A P D L I I N E Q R M T L P C M Y     hGR

398 G L G E L G A A L L Q L V R R L Q A L R L E R E E Y V L L K     hERR1
316 G L L E L Y R A I L Q L V R R Y K K L K V E K E E F V M L K     hERR2
420 G M V E I F D M L L A T S S R F R M M N L Q G E E F V C L K     hER
641 D Q C K - - - H M L Y V S S E L H R L Q V S Y E E Y L C M K     hGR

428 A L A L A N S D S V H I E D E P R L W S S C E K L L H E A L     hERR1
346 A L A L A N S D S M Y I E N L E A V - Q K L Q D L L H E A L     hERR2
450 S I I L L N S G V Y T F S L S S T L - K S L E E K D H I H R     hER
668 T L L L L S S V P K D G L - - - - - K S Q E L F D E I R M     hGR

458 L E Y E - - - - - - - - - A G R A G P G G G A E R R R A G R L   hERR1
375 Q D Y E - - - - - - - - - - L S Q R H E E E P R R A G K L L   hERR2
479 V L D K I T D T L I H L M A K A G L T L Q Q Q H Q R L A Q L L   hER
692 T Y I K - - - - E L G K A I V K R E G N S S Q N W Q R F Y Q L L   hGR

480 L L T L P L L R Q T A G K V L A H F Y G V K L E G K V P M H   hERR1
393 L L T L P L L R Q T A A K A V Q H F Y S V K L Q G K V P M W   hERR2
509 L L I L S H I R H M S N K G M E H L Y S M K C K N V V P L Y   hER
719 T K L L Q D S M H E V V V E Q L L N Y C F Q T F L D - - K T M S   hGR

510 K L F L E M L E A M M D •                            hERR1
423 K L F L E M L E A A K V •                            hERR2
539 Q L L L E M L D A H R L                 48 residues  hER
749 I E F P E M L A E I I T                 19 residues  hGR
```

FIG. V-3

**SUBSTITUTE SHEET**
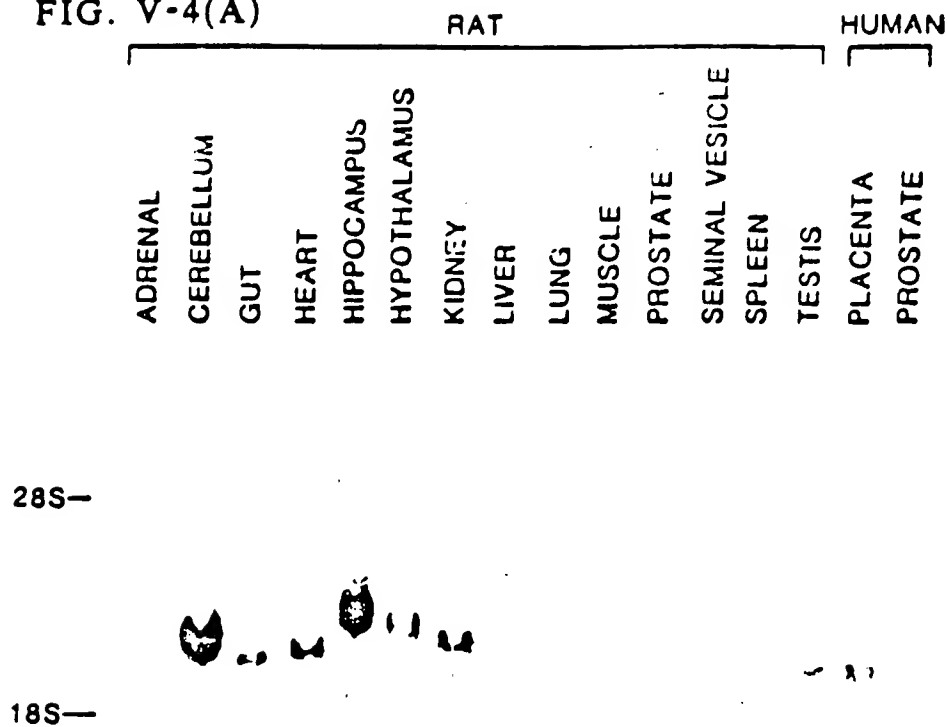
FIG. V-4(A)　　　　　　　　　　　RAT　　　　　　　　　HUMAN

ADRENAL
CEREBELLUM
GUT
HEART
HIPPOCAMPUS
HYPOTHALAMUS
KIDNEY
LIVER
LUNG
MUSCLE
PROSTATE
SEMINAL VESICLE
SPLEEN
TESTIS
PLACENTA
PROSTATE

28S—

18S—

FIG. V-4(B)

28S—

18S—

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FIG. V-5

FIG. VI-1(A)

FIG. VI-1(C)



| | Fold Induction by T₃ |
|---|---|
| C235 | 4.6 ± 0.4 |
| C181 | 2.6 ± 0.4 |
| C107 | 0.9 ± 0.5 |
| TK5 | 1.0 ± 0.2 |
| CTK5 | 2.5 ± 0.2 |
| COTK5 | 2.3 ± 0.2 |
| 90TK | 9.3 ± 2.2 |

FIG. VI-1(B)

| | Fold Induction by T₃ |
|---|---|
| TK4 | 1.2 ± 0.1 |
| CTK4 | 9.5 ± 0.2 |
| CΔ177-168 | 1.1 ± 0.1 |
| C29TK | 2.7 ± 0.4 |
| C293TK | 4.9 ± 0.6 |
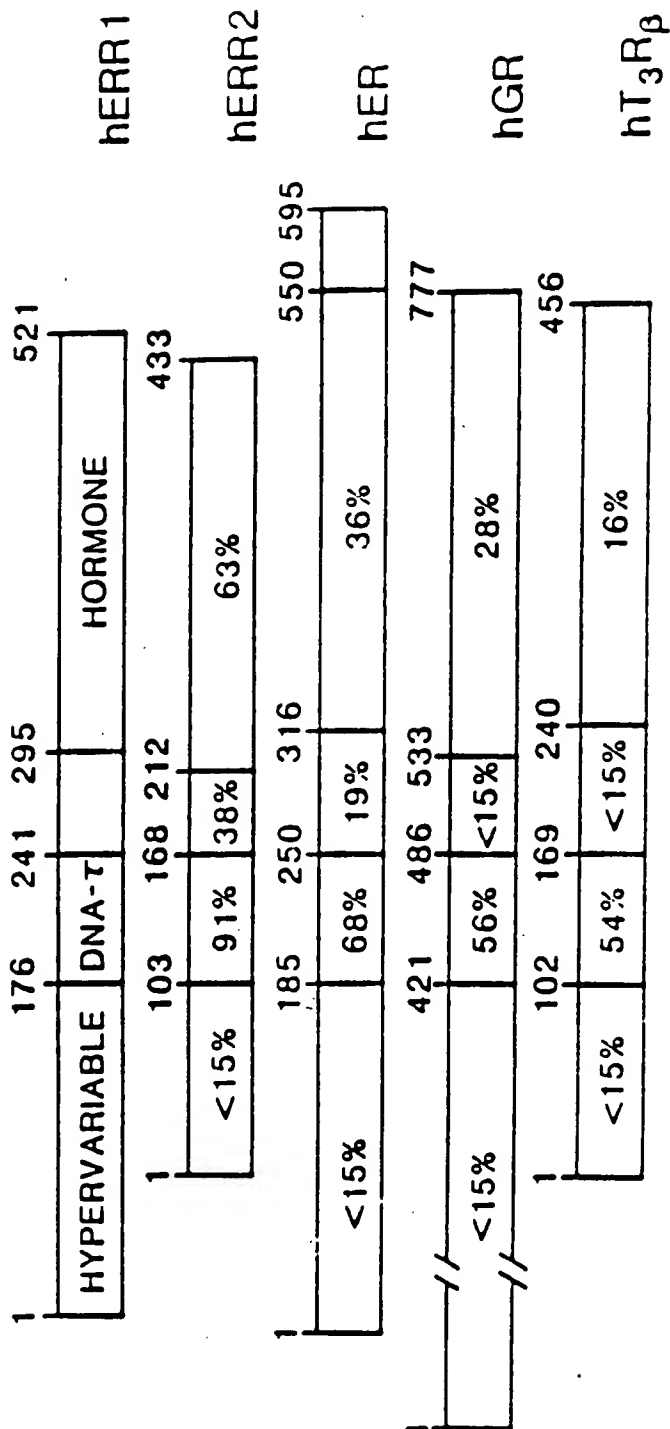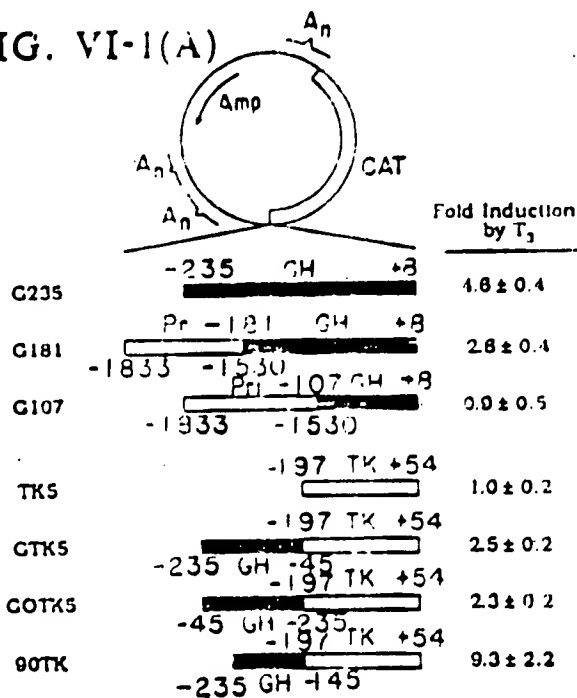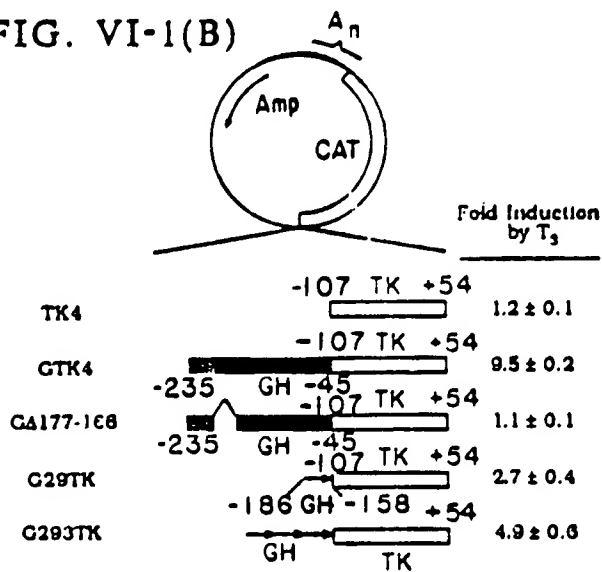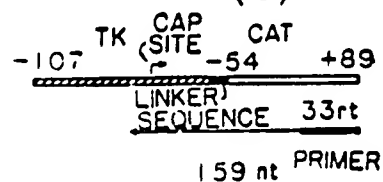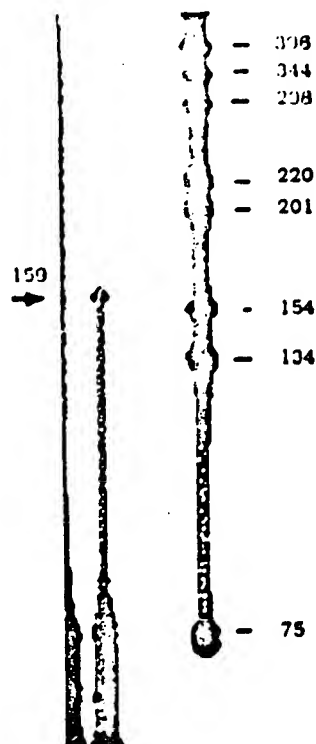
42/49
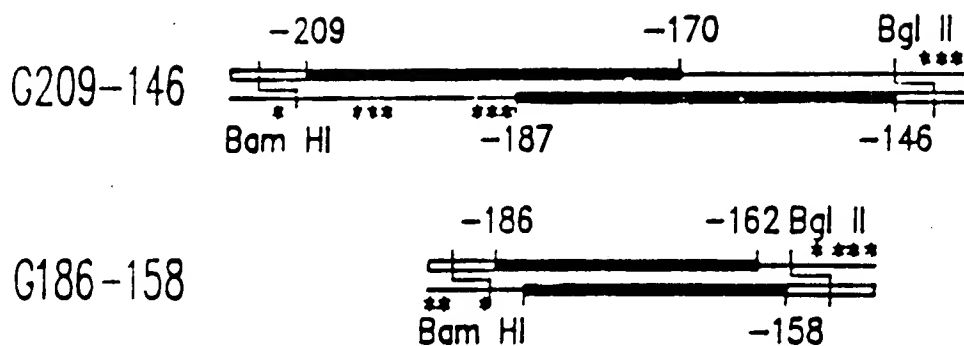
## FIG. VI-2(A)



## FIG. VI-2(B)



**SUBSTITUTE SHEET**

FIG. VI-3

A B C D E F G



178 —
163 —
140 —

110 —
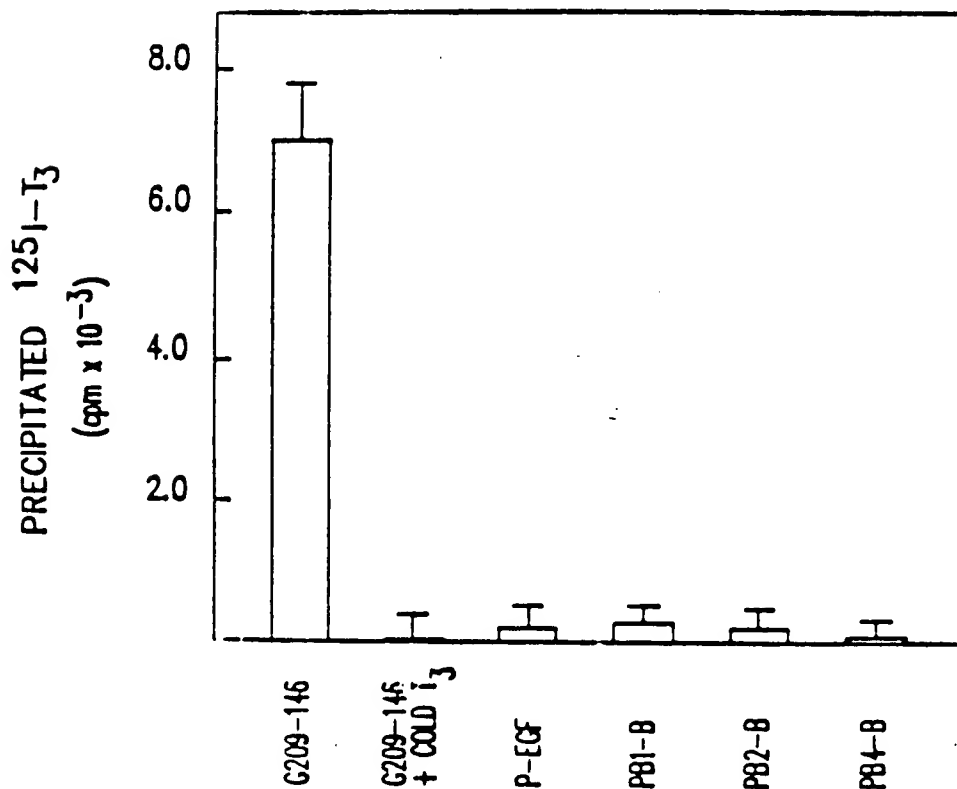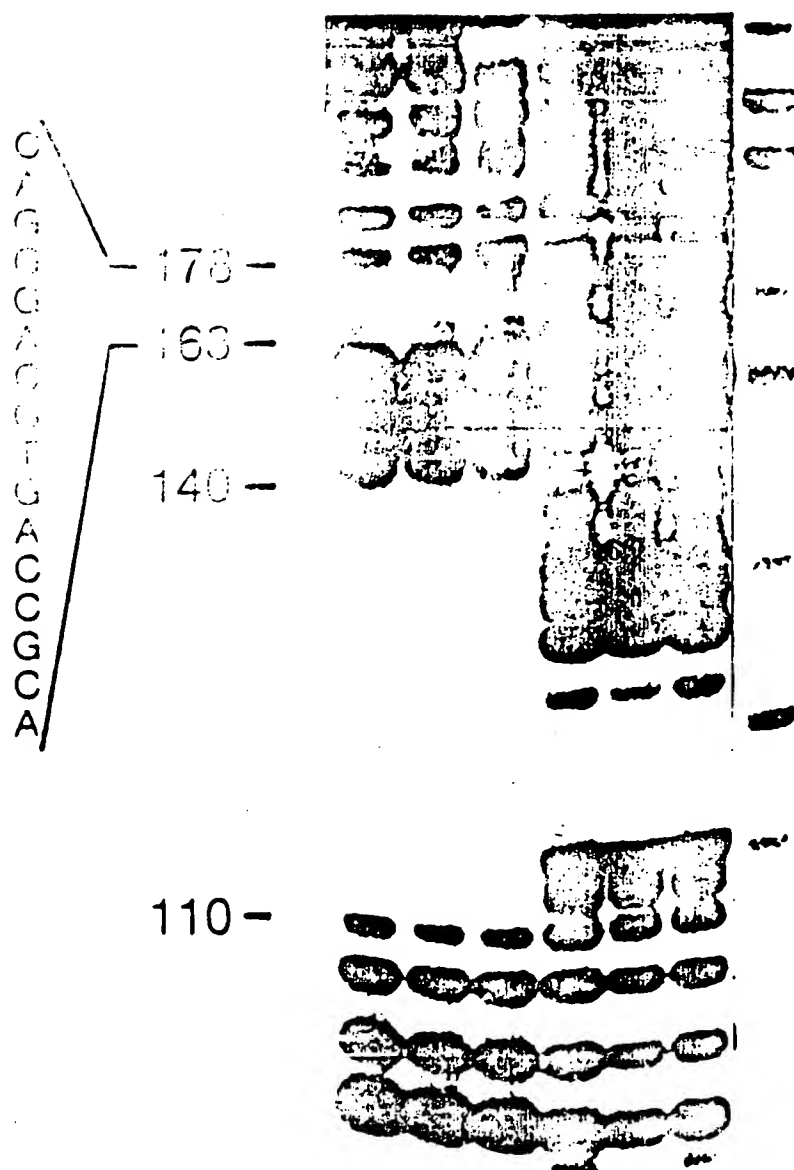
44/49

## FIG. VI-4

$^{125}$I–T$_3$–labeled T$_3$ receptor ☐

(cpm x 10$^{-3}$)

G209-146

G186-158

P-EGF

$^{35}$S–met hc erb A ▨

(cpm x 10$^{-3}$)

FIG. VII-1(A)

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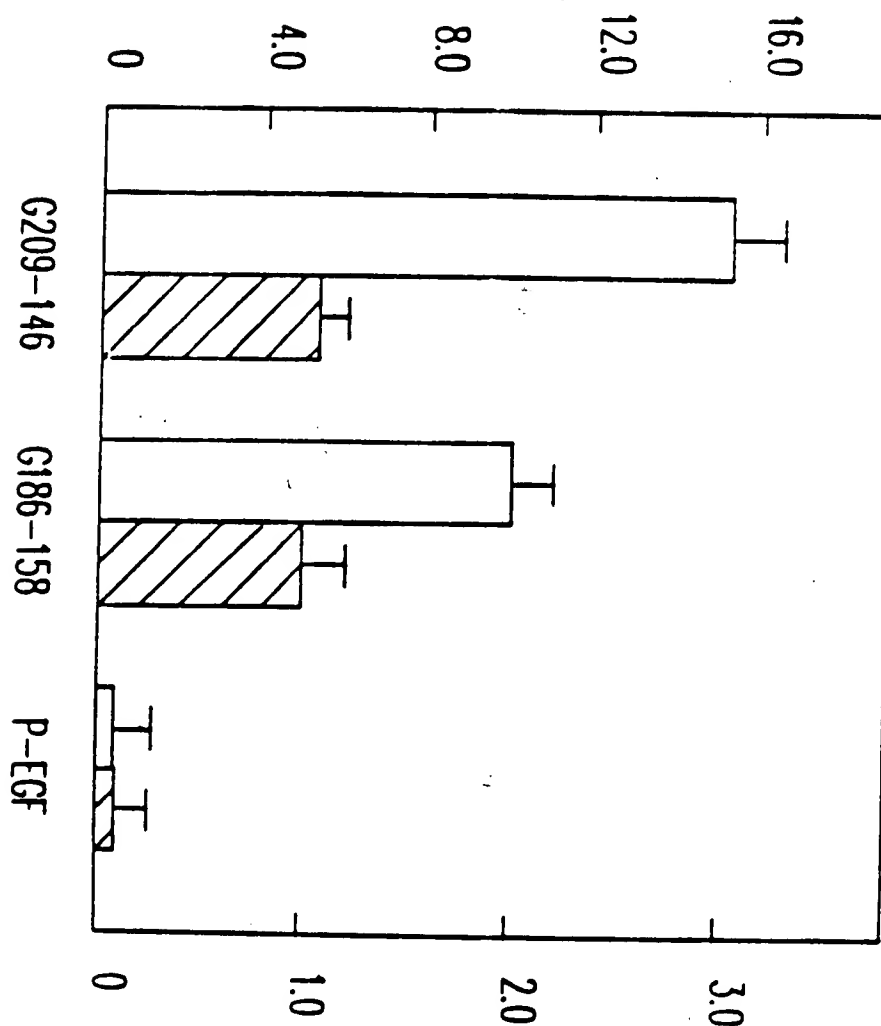


FIG. VII-1(B)

```
   1  CCAGCGCGCCAAGCCGAGGTATCTCCAGACAGGACAAGTCTCTGAAGACTTCCCAGCCCCCTAACCCAGTACCATT
  76  TACCAAGCCAGCTTGCCCCTAGCTCTCCCACCTGCCACTCACTCCCCAGCCCCCTCTAACCACCGGCCCTCCCGTT
 151  CTTTTTGGGGCACAGGGCATGGTGTGAAAGGCTGAGTACTGAGGGGGATACTATGGGTGCTGTCCCCTAGGGCCTG
 226  GGTGGCGGGGGGTGGGTGGCCTGTGGGCGTGGGGGGGGGCAGTGTGCCCACCCCAGTCTCTTGGCGTGCTGGAGG
                                                            MetGluGlnLysProSerLysValGluCysGlySerAspProGluGluAsn
 301  GCATCCCTGATGGAATTGAAGTGAATGGAACAGAAGCCAAGCAAGGTGGAGTGTGGGTCAGACCCAGAGGAGAAC
                            20              30              40
              SerAlaArgSerProAsnGlyLysArgLysArgLysAsnGlyGlnCysProLeuLysSerSerMetSerGlyTyr
 376  AGTGCCAGGTCACCAGATGGAAAGCGAAAAAGGAAGAACGGCCAATGTCCCCTGAAAAGCAGCATGTCAGGGTAT
                    50              60
            IleProSerTyrLeuAspLysAspGluGlnCysValValCysGlyAspLysAlaThrGlyTyrHisTyrArgCys
 451  ATCCCTAGTTACCTGGACAAAGACGAGCAGTGTGTCGTGTGTGGGGACAAGGCCACCGGTTATCACTACCGCTGT
                    70              80              90
            IleThrCysGluGlyCysLysGlyPhePheArgArgThrIleGlnLysAsnLeuHisProThrTyrSerCysLys
 525  ATCACTTGTGAGGGCTGCAAGGGCTTCTTTCGCCGTACAATCCAGAAGAACCTCCATCCCACCTATTCCTGCAAA
                    100             110
            TyrAspSerCysCysValIleAspLysIleThrArgAsnGlnCysGlnLeuCysArgPheLysLysCysIleAla
 601  TATGACAGCTGCTGCGTCATCGACAAGATCACCCGGAATCAGTGCCAGTTGTGTCGCCGCTTCAAGAAGTGCATCGCT
                    120             130             140
            ValGlyMetAlaMetAspLeuValLeuAspAspSerLysArgValAlaLysArgLysLeuIleGluGlnAsnArg
 675  GTGGGCATGGCCATGGACCTGGTTCTAGACGATTCAAAGCGGGTGGCCAAACGCAAGCTGATTGAGCAGAACCGG
                    150             160
            GluArgArgArgLysGluGluMetIleArgSerLeuGlnGlnArgProGluProThrProGluGluTrpAspLeu
 751  GAGAGGAGGCGAAAGGAGGAGATGATCCGCTCGCTGCAGCAACGACCAGAGCCCACTCCTGAAGAGTGGGATCTG
                    170             180             190
            IleHisValAlaThrGluAlaHisArgSerThrAsnAlaGlnGlySerHisTrpLysGlnArgArgLysPheLeu
 825  ATCCACGTTGCTACAGAGGCCCATCGCAGCACCAATGCCCAGGGCAGCCATTGGAAACAGAGGCGAAAATTCCTG
                    200             210
            ProAspAspIleGlyGlnSerProIleValSerMetProAspGlyAspLysValAspLeuGluAlaPheSerGlu
 901  CCCGGATGACATTGGCCAGTCACCTATTGTCTCCATGCCGGACGGAGACAAGGTGGACCTAGAGGCCTTCAGCGAG
                    220             230             240
            PheThrLysIleIleThrProAlaIleThrArgValValAspPheAlaLysLysLeuProMetPheSerGluLeu
 975  TTTACCAAGATCATCACCCCCGGCCATCACCCGCGTGGTGGACTTTGCCAAAAAACTGCCCATGTTCTCCGAGCTG
                    250             260
            ProCysGluAspGlnIleIleLeuLeuLysGlyCysCysMetGluIleMetSerLeuArgAlaAlaValArgTyr
1051  CCCTGTGAAGACCAGATCATCCTCCTGAAGGGCTGCTGCATGGAGATCATGTCCCTGCGGGCAGCTGTCCGCTAT
                    270             280             290
            AspProGluSerAspThrLeuThrLeuSerGlyGluMetThrValLysArgLysGlnLeuLysAsnGlyGlyLeu
1126  GACCCTGAGAGCGACACCCTGACCCTGAGTGGGGAGATGACGGTTAAGCGGAAGCAGCTCAAGAATGGTGGCTTG
                    300             310
            GlyValValSerAspAlaIlePheGluLeuGlyLysSerLeuSerAlaPheAsnLeuAspAspThrGluValAla
1201  GGTGTAGTCTCCGACGCCATCTTTGAACTGGGCAAGTCACTCTCTGCCTTTAACCTGGATGATACGGAAGTGGCT
                    320             330             340
            LeuLeuGlnAlaValLeuLeuMetSerThrAspArgSerGlyLeuLeuCysValAspLysIleGluLysSerGln
1276  CTGCTGCAGGCTGTGCTGCTGTTAATGTCAACAGACCGCTCTGGCCTGCTGTGTGTGGACAAGATCGAGAAGAGTCAG
                    350             360
            GlnAlaTyrLeuLeuAlaPheGluHisTyrValAlaAsnHisArgLysHisAsnIleProHisPheTrpProLysLeu
1351  GAGGCCTACCTGCTGGCGTTTGAGCACTACGTCAACCCGCGAAACACAACATTCCGCACTTCTGGCCCCAAGCTG
                    370             380             390
            LeuMetLysValThrAspLeuArgMetIleGlyAlaCysHisAlaSerArgPheLeuHisMetLysValGluCys
1426  CTGATGAAGGTGACTGACCTCCGGCATGATCGGGGGCCTGCCACGCCAGCCGCTTCCTCCACATGAAAGTCGAGTGC
                    400             410
            ProThrGluLeuPheProProLeuPheLeuGluValPheGluAspGlnGluValEnd
1501  CCCACCGAACTCTTCCCCCCACTCTTCCTCGAGGTCTTTGAGGATCAGGAAGTCTAAGCCTCAGGCGGCCAGAGG
1576  GTGTGCGGAGCTGGTGGGGAGGAGCTTGGAGAGAAGGACAAAGCTGGGGGTGAGGGAGAACCCCCATCTCTTC
1651  TCTCCTTCCTCCCGTCCTTGGATAGATACAGCTCCCATACACCCCTGCACCGCCCAGCCCCCTCAGAACCTCCAG
1726  CCCTTGGACAGGGCGACAAAATGAACTTGTTATGAAAGGCCGTGTGGGAGGCTGGGACCCAAGTTCTGAGTCTCTG
1801  GGACCCCAACCTCTAGAAAGTAGGGGAATGGAAGCAAAGACTGAGAGGGACAAGGACCATCTTGACTGTTGGGTCAGG
1876  AGGAATGAGGGAATAGGGCAGGTGCCCTTAATCACCCCTACACACACACAGGAGAGCCCCCCAAGCCCCTTGGCCT
1951  AGATTTCCCTTCAGGCCACAGGTCTCTACTTCCCCTAAATACCTGGGTCCTGGGTGCAAAGGACAGCTTGGCTTGG
2026  CTTAGCTCCTCCCCTGGAGGCTAAAAACGGTAGTCATCCTAACTGCAGTTTGCC   2079
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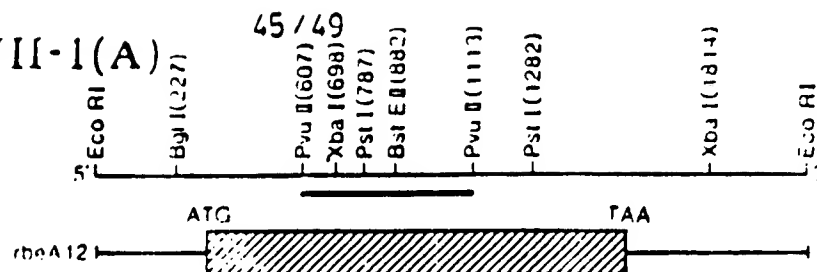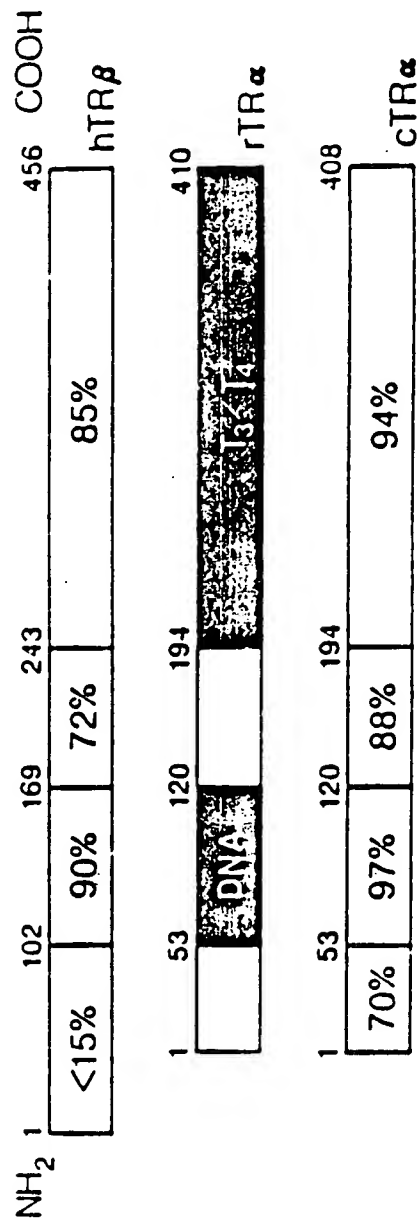
FIG. VII-2

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C. FIG. VII-3 (C)

B. FIG. VII-3(B)

A. FIG. VII-3(A)

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FIG. VII-4(C)

FIG. VII-4(B)

FIG. VII-4(A)

1  2  3

66K—

45K—

31K—

FIG. VII-5

# INTERNATIONAL SEARCH REPORT

**I. CLASSIFICATION OF SUBJECT MATTER** (if several classification symbols apply, indicate all) 3

According to International Patent Classification (IPC) or to both National Classification and IPC
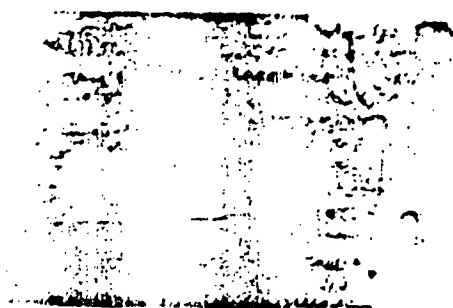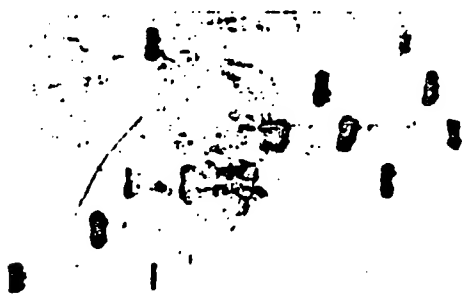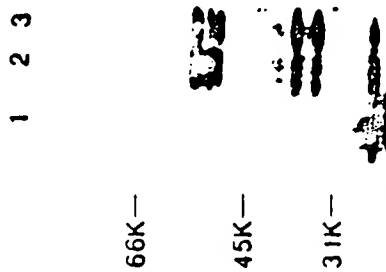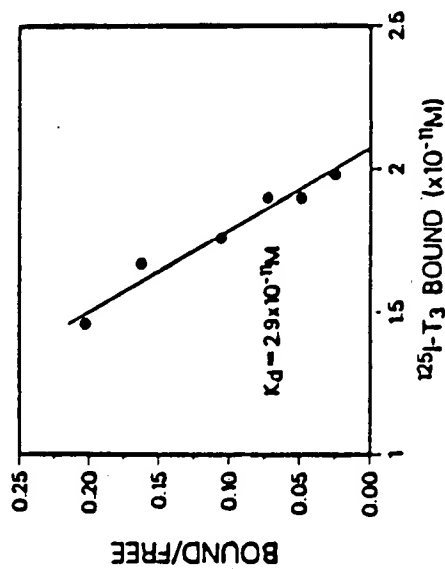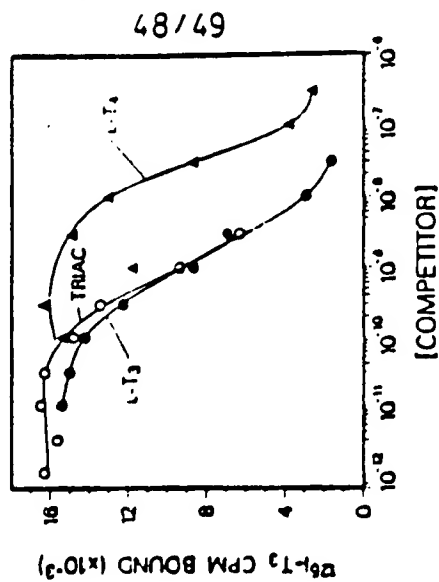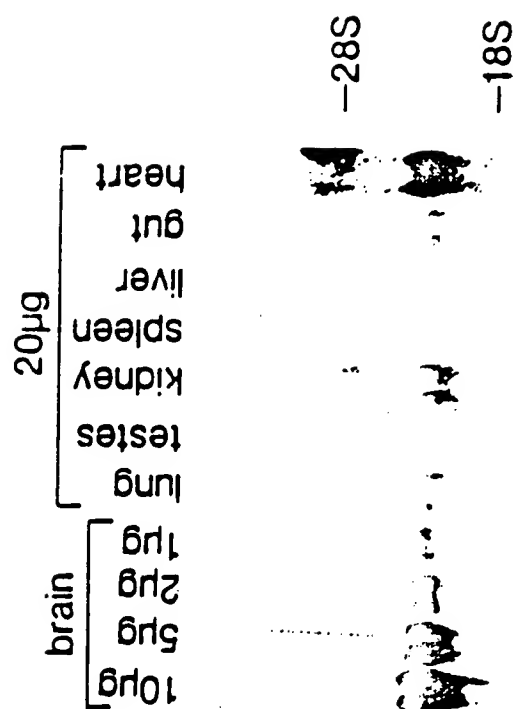
IPC(4): C12N 15/00; C12Q 1/68; C07K 13/00
US CL : 435/6, 172 3; 530/351

**II. FIELDS SEARCHED**

Minimum Documentation Searched 4

| Classification System | Classification Symbols |
|---|---|
| U.S. | 435/6,7,68,70,91,172.3,317;    530/350,351,820 935/13,29,38 |

Documentation Searched other than Minimum Documentation
to the Extent that such Documents are Included in the Fields Searched 5

CHEMICAL ABSTRACTS DATA BASE (CAS) 1967-1988 KEYWORDS:
HORMONE, RECEPTOR, DNA, cDNA, RNA, GLUCOCORTICOID,
MINERALOCORTICOID

**III. DOCUMENTS CONSIDERED TO BE RELEVANT** 14

| Category * | Citation of Document, 16 with indication, where appropriate, of the relevant passages 17 | Relevant to Claim No 18 |
|---|---|---|
| P,X | SCIENCE (Washington, D.C., U.S.A.), Volume 237, issued 17 July 1987 (ARRIZA ET AL), "Cloning of Human Mineralocorticoid Receptor Complementary DNA: Structural and Functional Kinship with the Gluco-corticoid Receptor", see page 268. | 1-46 |
| Y | THE JOURNAL OF BIOLOGICAL CHEMISTRY (Washington, D.C., U.S.A.), Volume 261, issued 25 August 1986, (NORTHROP ET AL), "Analysis of Glycocorticoid Unresponsive Cell Variants Using a Mouse Glucocorticoid Receptor Complementary DNA Clone", see page 11064. | 1-46 |
| Y | PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES (Washington, D.C., U.S.A.), Volume 81, issued August 1984, (NARAYAN ET AL), "Rapid induction of a specific nuclear mRNA precursor by thyroid hormone", see page 4687. | 1-27,34-36 |

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"&" document member of the same patent family

**IV. CERTIFICATION**

| Date of the Actual Completion of the International Search 1 | Date of Mailing of this International Search Report 1 |
|---|---|
| 1 February 1988 | 25 FEB 1988 |

| International Searching Authority 1 | Signature of Authorized Officer 16 |
|---|---|
| ISA/US | Stephanie Seidman, Ph.d., J.D. |

| Category* | Citation of Document, with indication, where appropriate, of the relevant passages | Relevant to Claim No. |
|---|---|---|
| Y | THE EMBO JOURNAL (Oxford, England), Volume 5, issued May 1986, (KRUST ET AL), "The chicken oestrogen receptor sequence: homology with v-erbA and the human oestrogen and glucocortcoid receptors", see page 891. | 1-27, 34-36 |
| Y | THE EMBO JOURNAL (Oxford, England), Volume 5, issued October 1986, (DANIELSEN ET AL), "The mouse glucocorticoid receptor: mapping of functional domains by cloning, sequencing, and expression of wild-type and mutant receptor proteins", see page 2513. | 1-46 |
| Y | THE EMBO JOURNAL (Oxford, England), Volume 5, issued September 1986, (CATO ET AL), "The hormone regulatory element of mouse mammary tumour virus mediates progesterone induction", see page 2237. | 34-46 |
| Y | MOLECULAR AND CELLULAR BIOLOGY (Washington, D.C., U.S.A), Volume 3, issued July 1983, (DANIELSEN ET AL), "Immunological Selection of Variant Mouse Lymphoid Cells with Altered Glucodorticoid Responsiveness", see page 1310. | 28-33 |
| Y | CELL (Cambridge, Massachusetts, U.S.A.), Volume 33, issued June 1983, (CHANDLER ET AL), "DNA Sequences Bound Specifically by Glucocorticoid Receptor In Vitro Render a Heterologus Promoter Hormone Responsive In Vivo", see page 489. | 34-36 |
| Y | CELL (Cambridge, Massachusetts, U.S.A), Volume 31, issued December 1982, (PFAHL), "Specific Binding of the Glucocorticoid-Receptor Complex to the Mouse mammary Tumor Proviral Promoter Region", see page 475. | 34-46 |
| Y | PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES (Washington, D.C., U.S.A), Volume 79, issued September 1982, (GOVINDAN ET AL), "Purified glucocorticoid receptor-hormone complex from rat liver cytosol binds specifically to cloned mouse mammary tumor virus long terminal repeats in vitro", see page 5157. | 1-46 |

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**III. DOCUMENTS CONSIDERED TO BE RELEVANT** (CONTINUED FROM THE SECOND SHEET)

| Category * | Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹² | Relevant to Claim No ¹³ |
|---|---|---|
| Y | PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES (Washington, D.C., U.S.A.), Volume 82, issued February 1985, (MOORE ET AL), "The first intron of the human growth hormone gene contains a binding site for glucocorticoid receptor", see page 699. | 1-46 |
| Y | BIOCHEMISTRY (Washington, D.C., U.S.A.) Volume 23, issued 18 December 1984, (MILLER ET AL), "Covalent and Non-covalent Receptor - Glucocorticoid Complexes Preferentially Bind to the Same Regions of the Long Terminal Repeat of Murine Mammary Tumor Virus Proviral DNA", see page 6883. | 1-46 |
| Y | NATURE (London, England), Volume 320, issued 13 March 1986, (GREEN ET AL), "Human oestrogen receptor cDNA: sequence, expression and homology to v-erb-A", see page 134. | 1-27, 34-46 |
| Y | MOLECULAR AND CELLULAR BIOLOGY, (Washington, D.C., U.S.A), Volume 6, issued April 1986, (DEFRANCO ET AL), "Two Different Factors Act Separately or Together to Specify Functionally Distinct Activities at a Single Transcriptional Enhancer, see page 993. | 28-46 |
| Y | NATURE, (London, England), Volume 312, issued 20/27 December 1984, (MIESFILD ET AL), "Characterization of a steriod hormone receptor gene and mRNA in wild-type and mutant cells", see page 779. | 1-27, 34-46 |
| Y | MOLECULAR AND CELLULAR BIOLOGY, (Washington, D.C., U.S.A.), Volume 4, issued June 1984, (FIRZLAFF ET AL), "Dexamethasone Increases the Number of RNA Polymerase II Molecules Transcribing Integrated Mouse Mammary Tumor Virus DNA and Flanking Mouse Sequences", see page 1057. | 28-46 |
| Y | THE EMBO JOURNAL, (Oxford, England), Volume 1, issued December 1984 (GEISSE ET AL), "Glucocorticoid receptors recognize DNA sequences in and around mammary tumour virus DNA", see page 1613. | 28-46 |

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